A Seminar Paper on

Recent Advancements in Rapid Detection of Fish Pathogens

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Submitted to:

Course Instructors	Major Professor
Dr. A. K. M. Aminul Islam Professor Department of Genetics and Plant Breeding **Dr. Satya Ranjan Saha** Professor Department of Agroforestry and Environment **Dr. Shaikh Shamim Hasan** Professor, Department of Agricultural Extension and Rural Development **Dr. Dinesh Chandra Shaha** Associate Professor, Department of Fisheries Management, BSMRAU	**Dr. Md. Mahbubur Rahman** Professor Institute of Biotechnology and Genetic Engineering, BSMRAU

Submitted by:

Ms Khadiza Sarkar

MS student, Reg. no: 17-05-4316

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Institute of Biotechnology and Genetic Engineering, BSMRAU

BANGABANDHU SHEIKH MUJIBUR RAHMAN AGRICULTURAL UNIVERSITY

Salna, Gazipur – 1706

Recent Advancements in Rapid Detection of Fish Pathogens[1]

by

Ms Khadiza Sarkar[2]

ABSTRACT

Rapid detection of fish pathogens ensures timely medication and prevents disease outbreaks in farms reducing harmful consequences of antibiotics. Traditional and PCR based diagnostic assays fail in rapid detection leading to late action. Dependence on sophisticated laboratory facilities with skilled manpower limits their application in remote level. In 2014, researchers developed a novel real time isothermal recombinase polymerase amplification (RPA) assay to detect white spot syndrome virus (WSSV) in shrimp with detection level up to 10 molecules in 95% of cases within 6.41 ± 0.17 min at 39°C. Researchers have also adopted the CRISPR-based sensitive high efficiency reporter unlocking (SHERLOCK) method in 2019 and made it more rapid and effective by combining lateral flow colorimetric reporting and paper matrix based nucleic acid extraction that made detection of WSSV possible within just 1 hour in the pond side with highest sensitivity. Most recently in 2022, to avoid the high cost and complexity of SHERLOCK principle, combination of RPA with lateral flow strip was designed with sensitivity of 20 copies/reaction performing at 37°C. In this paper these recent molecular techniques are reported and the efficacies discussed and comparison of efficacies found the SHERLOCK assay the most feasible one as it is highly sensitive and fully field deployable. The limitations here are, real time RPA needs extra scanning, SHERLOCK is costly for the farmer and RPA-LFS is easy but less sensitive. This review may contribute to adopt these methods for other fish pathogens.

Keywords: rapid detection, fish pathogen, white spot syndrom virus, real time polymerase chain reaction, recombinase polymerase amplification, sensitive high efficiency reporter unlocking, lateral flow strip.

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[2] Ms Student, Institute of Biotechnology and Genetic Engineering, BSMRAU, Gazipur 1706

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Chapter I

INTRODUCTION

Aquaculture is an important contributor to the global food supply (Charoonnart *et al.*, 2018) and playing crucial role in the economy of Bangladesh as well as other countries (Ahmed, 2013; Dey *et al.*, 2008). In Bangladesh, about 1.2 million people find jobs in inland water fishing, while another 0.3 million people find jobs in marine fishing. The fisheries sector is currently contributing 3.52 percent of the GDP. Fish provide 55 percent of animal protein intake in Bangladesh (Hossain *et al*., 2014).

One of the greatest threats to the growth of aquaculture production is the spread of infectious diseases. Due to high stocking densities, increased stress, and inadequate water exchange, fish become more susceptible to various infectious diseases. While some pathogens cause growth retardation, others can cause varying level of mortality. For example, white spot syndrome virus in shrimp is so lethal that it can cause 100% mortality in just 1 week. Outbreaks of infectious diseases regularly affect shrimp farms, resulting in loss of reared shrimp and limiting both domestic and global production to 15 billion dollars per disease and over 20 billion dollars in total. So, large-scale efforts are needed to develop diagnostic tools to support early detection and mitigate the spread of these pathogens (Lightner *et al*., 2012).

Rapid and specific detection of a disease is an integral part of fish health management. Farmers cannot instantly diagnose the specific disease and thus apply different antibiotics from their practical experiences. This practice creates greater problems like within that period the disease already spreads all over the farm or area and can cause mass mortality as well as economic loss. Another long lasting effect of that indiscriminate application of antibiotics is increased chance of development of resistant strains of bacteria in that culture system, along with residual effects in water if with-drawl period is not maintained properly (Cabello, 2006; Defoidt *et al.*, 2011). Moreover, the antimicrobial agents used in the farms are released to the surrounding water-bodies (Bjorklund *et al.*, 1990). As a result a high incident of bacteria resistant to the antimicrobials used in aquaculture have been detected in the culture ponds and surrounding aquatic environments (McPHearson *et al*., 1991; Sandaa *et al.*, 1992; DePaola *et al*., 1995; Schmidt *et al.*, 2000) that are used for daily household purposes by the farm workers and local people and works as threats to the environment and public health. Such incidents increase the potential risk that antibiotic resistance genes could be spread into a wide range of aquatic bacteria (Petersen *et al*., 2002) and it has been linked to certain

antimicrobial resistance patterns among human bacterial pathogens (Bager *et al*., 1997; Wegener *et al*., 1999) indicating a possible flow of antimicrobial resistance genes between fish and human pathogens.

The ultimate solution for all these problems is correct and rapid detection of the fish pathogen as soon as any symptoms are noticed before the disease spreads. As we know prevention is better than cure, so this detection of pathogen can also be used while we are stocking water and seeds to the pond to ensure both are specific pathogen free. So, availability of rapid, sensitive and cost effective diagnostics to both in the hatcheries and fish farm are crucial for limiting disease outbreaks.

However, at present fish disease diagnosis in Bangladesh is still dependent on signs and symptoms and the first ever aquatic animal disease diagnostic laboratory of Bangladesh has been founded in Khulna in February 2021 by WorldFish Bangladesh jointly with Fishtech (BD). This laboratory is equipped with real-time PCR and can detect only 11 types of harmful viruses and bacteria in fish and shrimp. From last two decades different PCR dependent assays and fluorescence in situ hybridization are being used as standard methods for disease detection in the world. But these assays are highly dependent on sophisticated laboratory facilities. Therefore, there is an urgent need for new methodologies that are inexpensive and rapid, yet highly sensitive, enabling point-of-care testing in the field particularly in developing and underdeveloped nations where limited infrastructure exist but aquaculture is growing rapidly.

This study aims to consolidate the recent advancements happened in rapid detection of fish pathogen and updates our understanding on the potentials of these new technologies.

Objectives

The specific objectives of this review paper are as follows:

1. To report the recent methods developed for rapid detection of fish pathogens
2. To compare the efficacy, feasibility and limitations of different advanced methods developed for rapid detection of fish pathogens

Chapter II

MATERIALS AND METHODS

This seminar paper is a review paper based on secondary information. Different published articles are used to prepare this paper. Information has been assembled from various articles published in the journals, book, and websites available on the online platform.

Constructive suggestions from my major professor and course instructors helped me to improve this paper. After the collection of all the related information, it was gathered and logically presented in the current form.

Chapter III

REVIEW OF FINDINGS

In this chapter, findings of different authors have been accumulated and discussed under different headings to ascertain the objectives of the paper.

3. Recent rapid diagnostic methods used in fish pathogen identification

Detection of fish pathogen needs three basic steps to be performed.

1. Extraction of the nucleic acid of the pathogen from the fish sample.
   Common Techniques used are manual DNA/ RNA extraction method or different commercial DNA/ RNA extraction kits or recent paper matrix based nucleic acid extraction method.
2. Amplification of the extracted nucleic acid with specifically designed primers through any of the amplification assay combining with fluorescence or chemical probe to visualize the amplified nucleic acids. Techniques available are: different PCR based molecular methods, Recombinase polymerase amplification (RPA), CRISPR- based methods etc.
3. Read amplification result or confirm the presence or absence of the target pathogen in the sample through visualizing the fluorescence probe using scanner or chemical probe using lateral flow colorimetric reporting etc.

Recently used rapid detection methods for fish pathogen are combination of the advanced techniques from the above steps. The efficacy of any rapid diagnosis method depends on few parameters. These are:

1. Time required to amplify the nucleic acids of the pathogen (determine the rapidity of the method)
2. Temperature at which the amplification assay is performed (Isothermal assays especially the temperature near to room temperature ensure usability out of lab)
3. Sensitivity of the method (lowest limit of the copy number of the pathogen's nucleic acid that can be detected)
4. Reliability of the method (whether the rapid method provides similar valid results compared to a standard assay)

5. Specificity of the method (how much specific the method is in detecting the target pathogen to reduce false positive results created by other pathogen presence)
6. Laboratory equipment dependency or on-site detection ability
7. Complexity of the method (designing the specific primers, probes, guide RNAs etc.)
8. Cost of the method

Here three distinct combinations have been found potential by scientist to detect the white spot syndrome virus (WSSV) in shrimp in a rapid, field deployable way. The efficacy of each of these three techniques will be discussed on the basis of the above described parameters with proper data comparing with standard real time PCR method. Then the feasible one will be declared by comparing the efficacy of these three rapid detection methods for fish pathogen.

3.1 Real time isothermal recombinase polymerase amplification (RPA) assay

qPCR is an standard assay used for disease detection due to its superior sensitivity but it requires expensive thermal cycler, time consuming, labor intensive and depends on gel electrophoresis to read amplified results that makes it highly laboratory dependent. For pond side detection isothermal LAMP use simple heating block but the amplified products are not amenable for quantification and LAMP primer design is complex. So, RPA is a good option here. RPA couples isothermal recombinase-driven primer targeting the template with strand-displacement DNA synthesis (Pipenburg *et al.*, 2006). In this particular assay, the techniques used in the three basic steps of pathogen detection are listed in Table 1.

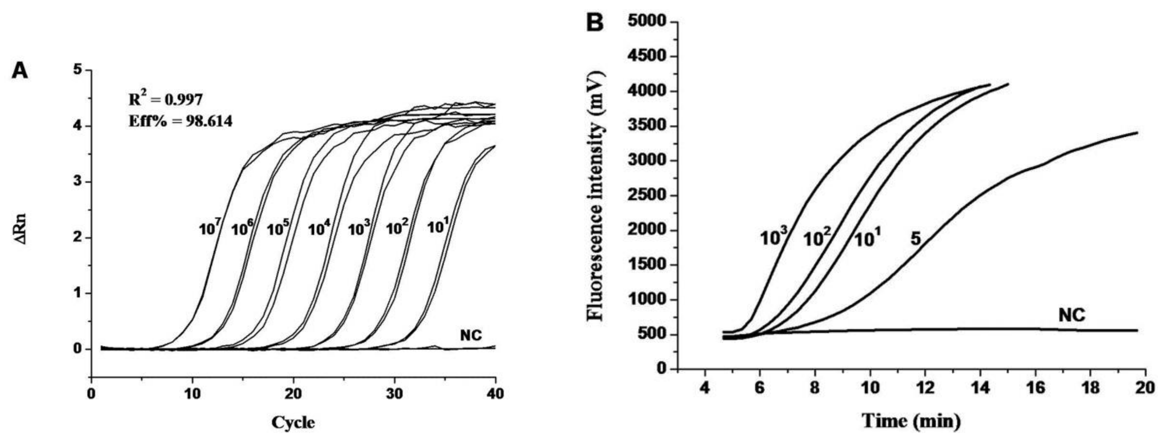
Table 1. Techniques used in real-time isothermal recombinase polymerase amplification assay

DNA Extraction	Amplification assay	Confirmation assay
TIANamp Marine Animals DNA kit	Real-time RPA using Twist Amp exo kit	ESEQuant Tube scanner device

Source: Xia *et al.*, 2014

3.1.1 Sensitivity and specificity of the real-time RPA assay

Figure 1A shows that qPCR have sensitivity of detecting WSSV DNA standard plasmids at least 10 copies per reaction on the other hand in figure 1B real-time RPA can detect at least 5 molecules per reaction. Negative control showing very low fluorescence intensity proves the specificity of both of these assays. As RPA can detect a limit up to 5 copies within 7.12±0.50 min so it is a rapid method with high sensitivity.



Source: Xia *et al.*, 2014

Figure 1. Amplification curve of qPCR (1A) and real-time RPA (1B) describing Sensitivity, specificity of real-time RPA

3.1.2 Reliability of real-time RPA assay

To confirm the stability of the RPA assay, 34 shrimp individuals were tested to detect WSSV infection. In both RPA and qPCR assay, 22 shrimps found WSSV positive, and 11 negative. But one shrimp sample was showing weak positive amplification signal in RPA assay, but negative in qPCR. So, RPA for detecting WSSV turned out to be reliable. (Xia *et al.*, 2014)

3.1.3 The efficacy of real-time RPA method is described by comparing with ideal qPCR method based on different parameters in Table 2.

Table 2: Efficacy of real-time isothermal recombinase polymerase amplification assay

Method	Reaction Time min/C_T	Temperature(°C)	Sensitivity (Copies/reaction)	Reliability	Specificity	Lab dependency	Complexity
real-time RPA	7	39	5	87.5 %	100%	no	Primers deign complex
qPCR	30	60-94	12	95%	100%	yes	Primers available

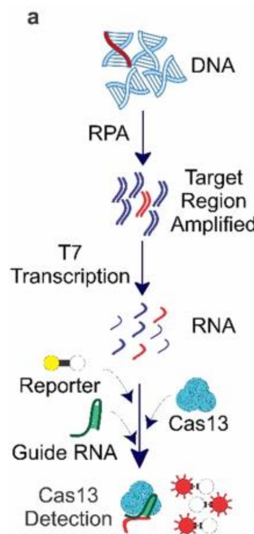
Source: Xia *et al.*, 2014

3.2 Rapid, CRISPR based, field-deployable SHERLOCK assay

In the previously used real time RPA method, ESEQuant Tube scanner device is required to be connected with a computer to measure the fluorescence intensity. But it is not available every now and then for a marginal fish farmer. Again DNA extraction was done by

commercial kit in the above assay which also need more or less 1.5 hour. So, combinations of a rapid on-spot DNA extraction tool will faster this whole detection method. Using CRISPR combined with RPA also helps to increase the sensitivity of the assay. So, scientist came with this CRISPR based field deployable diagnostic method called sensitive high efficiency reporter unlocking (SHERLOCK)

In 2017, Gootenberg and colleagues cobined the collateral ribonuclease activity of Cas13a with isothermal amplification to create a diagnostic test called SHERLOCK for detection of human pathogens (Gootenberg *et al.*, 2017). The SHERLOCK method starts with isothermal amplification using RPA, followed by T7 transcription to produce RNA from amplified copies, and at last Cas13a detection to enable fluorescent or color dependent detection (Fig. 2). Scientist later adopted this technique for rapid detection of WSSV in shrimp by designing primers and developing a guide RNA probe. This adaption provides point-of care detection at the pond site.



Source: Sullivan *et al.*, 2019

Figure 2. Principle of SHERLOCK method

In this particular assay, the techniques used in the three basic steps of pathogen detection are listed in Table 3.

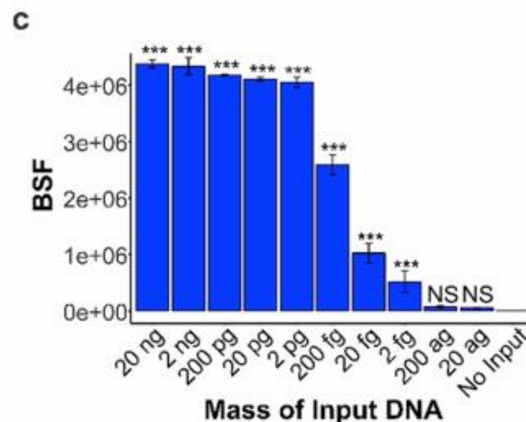
Table 3: Techniques used in SHERLOCK assay

DNA Extraction	Amplification assay	Confirmation assay
Standard column DNA extraction	CRISPR-based SHERLOCK method	Lateral flow colorimetric reporting

Source: Sullivan *et al.*, 2019

3.2.1 Sensitivity of SHERLOCK method:

To measure the sensitivity of this assay they measured the limit of detection of this assay by taking 20 ng of DNA from a single infected sample and conducting a 10-fold serial dilution down to 20 attograms of input DNA. The SHERLOCK method managed to detect down to 2 femtograms of input DNA which based on the copy number of the individual (~530,000 copies ng DNA) was ~1.06 copies or single copy detection proving high sensitivity (Fig. 3).



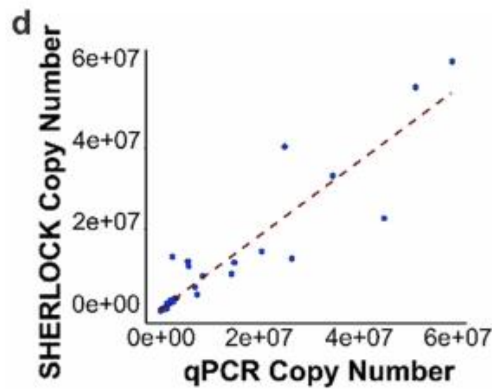
Source: Sullivan *et al.*, 2019

Figure 3. Limit of detection analysis for the SHERLOCK assay using a diluted positive shrimp sample

Here, reaction input ranges from 20 ng of DNA to 2 ag of DNA translating to 10,600,000 to 0.001 copies per reaction. NS = not significant, *p <0.05, **=p<0.01, ***p<0.001 based on comparisons to no input control from ANOVA and Tukey's post-hoc results. Error bars denote standard deviation. BSF indicates background subtracted fluorescence.

(3.2.2) Reliability of SHERLOCK method:

35 WSSV infected *P. vannamei* samples were tested from experimental challenges with Sherlock and OIE (The World Organization for Animal Health, Paris, France)-recommended qPCR alongside standard curves to quantify and compare WSSV copy number determined by these two methods. Infection was detected in all positive samples and the two approaches showed strong correlation in the copy numbers estimated for each sample (Fig.4).



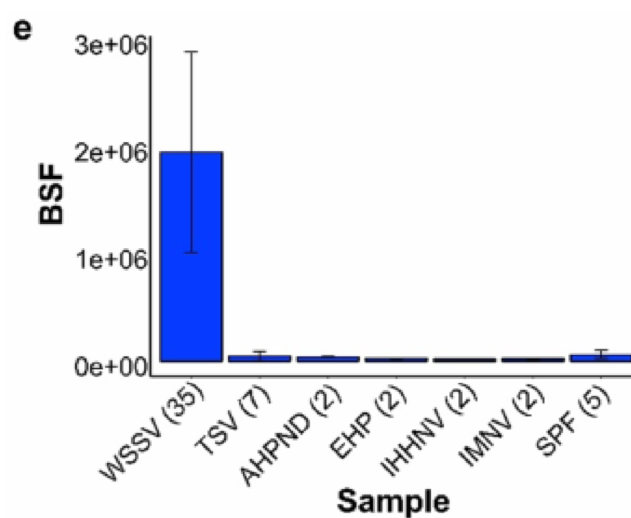
Source: Sullivan *et al.*, 2019

Figure 4. Comparison of WSSV copy number detected by qPCR and SHERLOCK assays.

As the correlation between the results provided by both qPCR and SHERLOCK assay is strong (r =0.93 which was significant p= 1.4e-15) so, SHERLOCK assay is highly reliable.

3.2.3 Specificity of SHERLOCK method:

A number of individuals infected with other common shrimp pathogens (Acute Hepatopancreatic Necrosis Diseases [AHPND] caused by *Vibrio parahaemolyticus Enterocytozoon hepatopenaei* [EHP], Infectious Hypodermal and Hematopoietic Necrosis Virus [IHHNV], Infectious Myonecrosis Virus [IMNV], and Taura Syndrome Virus [TSV] as well as verified Specific Pathogen Free [SPF] *P. vannamei* samples. In all cases, the SHERLOCK assay showed no positive detection (Fig. 5).



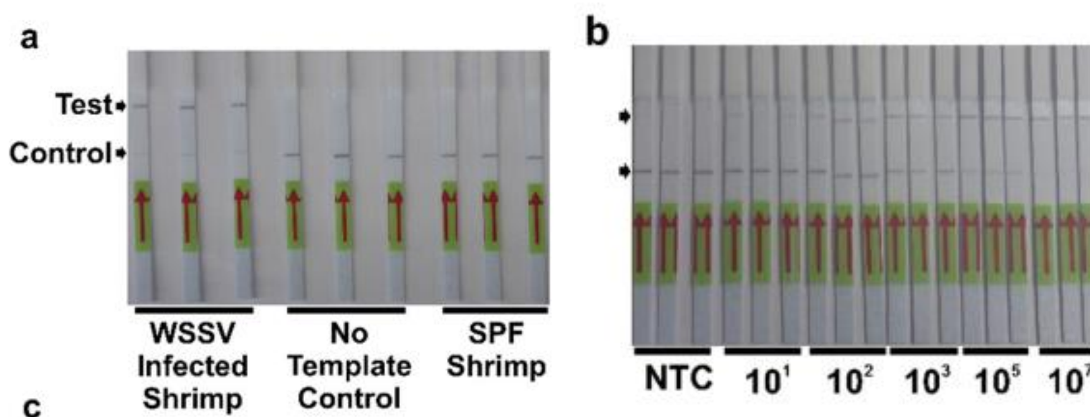
Source: Sullivan *et al.*, 2019

Figure 5. Specificity tests of SHERLOCK assay evaluating detection of WSSV with other common shrimp disease.

All these results show that SHERLOCK assay is highly accurate diagnostic, possessing exceptional diagnostic sensitivity, analytical sensitivity, and analytical specificity for use in detecting WSSV infecting marine crustaceans.

3.2.4 Combining lateral flow colorimetric reporting with SHERLOCK method to make it fully field deployable:

To make this SHERLOCK method field deployable, choosing a colorimetric method over fluorescence is necessary. So lateral flow “hybridetect” testing strips from Milenia Bitec with a custom reporter for colorimetric reaction was used. To ensure the efficacy of this Lateral flow assay to detect WSSV the specificity, and sensitivity were evaluated (Fig. 6 a & 6 b).



Source: Sullivan *et al.*, 2019

Figure 6. Specificity (6a), and sensitivity (6b) of lateral flow assay for WSSV

3.2.5 Specificity of the lateral flow assay

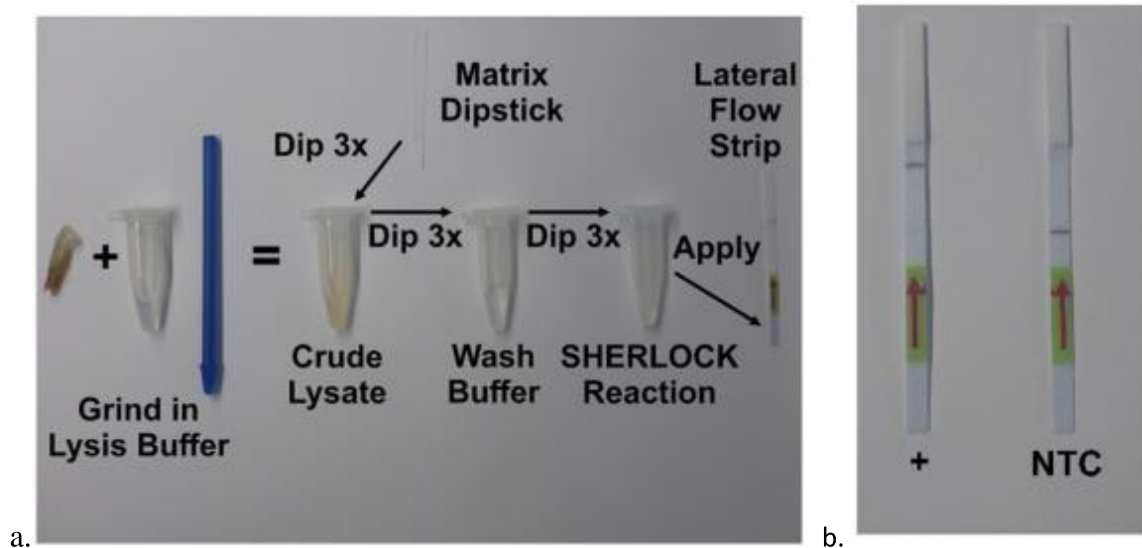
The lateral flow assay accurately detected WSSV infected experimentally challenged shrimp and showed no positive detection for No Template control reactions or Specific Pathogen Free (SPF) shrimp samples (Fig. 6 a). So, this assay is highly specific and can reliably replace the Fluorescent based assay that was depended on electronic detection device.

3.2.6 Sensitivity of the lateral flow assay

Diluted synthetic target DNA of known copy number was tested for sensitivity evaluation. The Lateral Flow Assay was able to detect as few as 10 synthetic DNA copies (Fig. 6 b).

3.2.7 Paper matrix based nucleic acid extraction

This method eliminates the dependence of complex equipment and facilitates the DNA extraction. This process takes advantage of the binding properties of cellulose fibers. 21 At first matrix dipsticks are prepared by dipping Whatman #1 filter paper in melted Paraplast (Sigma) (Zou *et al*., 2017). These dipsticks then dried by hanging and cut into thin strips. The extraction lysis buffer that was used is: 20 mM Tris [pH 8.0], 25 mM NaCl, 2.5 mM EDTA, and 0.05% SDS. The extraction method involved grinding 2mg of muscle tissue in lysis buffer, shaking the lysate, dipping the dipstick in the lysate 3 times, dipping the dipstick in wash buffer (10 mM Tris, 0.01% Tween-20) 3 times, and dipping the dipstick in the SHERLOCK reaction 3 times (Figure 7a). This was then incubated for 45 minutes. This paper matrix base nucleic acid extraction procedure was used to extract DNA from a positively experimentally challenged shrimp sample alongside no template controls. When the dipstick based extracted DNA tested with SHERLOCK method, all the WSSV positive samples was detected with strong signal.



Source: Sullivan *et al*., 2019

Figure 7. Schematic representation of combined matrix extraction and lateral-flow SHERLOCK reaction allowing rapid field-deployable diagnostic (7a), test results (7b)

By adopting the combination of matrix extraction and lateral flow SHERLOCK it was possible to extract amplify and detect viral DNA from experimentally challenged shrimp in approximately 60 minutes at room temperature. Strong positive signal showed in the strip (Fig.7b) for an individual with a copy number of 700,000 copies per ng of DNA.

Though primary assess and validation of Lateral Flow SHERLOCK assay's performance (i.e. diagnostic sensitivity, analytical sensitivity, and analytical specificity) was done using a standard column DNA extraction, it is now proved that combining Lateral flow SHERLOCK's exceptional properties with the paper matrix extraction makes the whole detection method fully field deployable. It requires no electricity, heat, advanced training, or specialized equipment. So it is a method that can be regularly used by aquaculture professionals, fish farmers under field condition.

3.2.8 The efficacy of lateral flow SHERLOCK method is described by comparing with ideal qPCR method based on different parameters in Table 4.

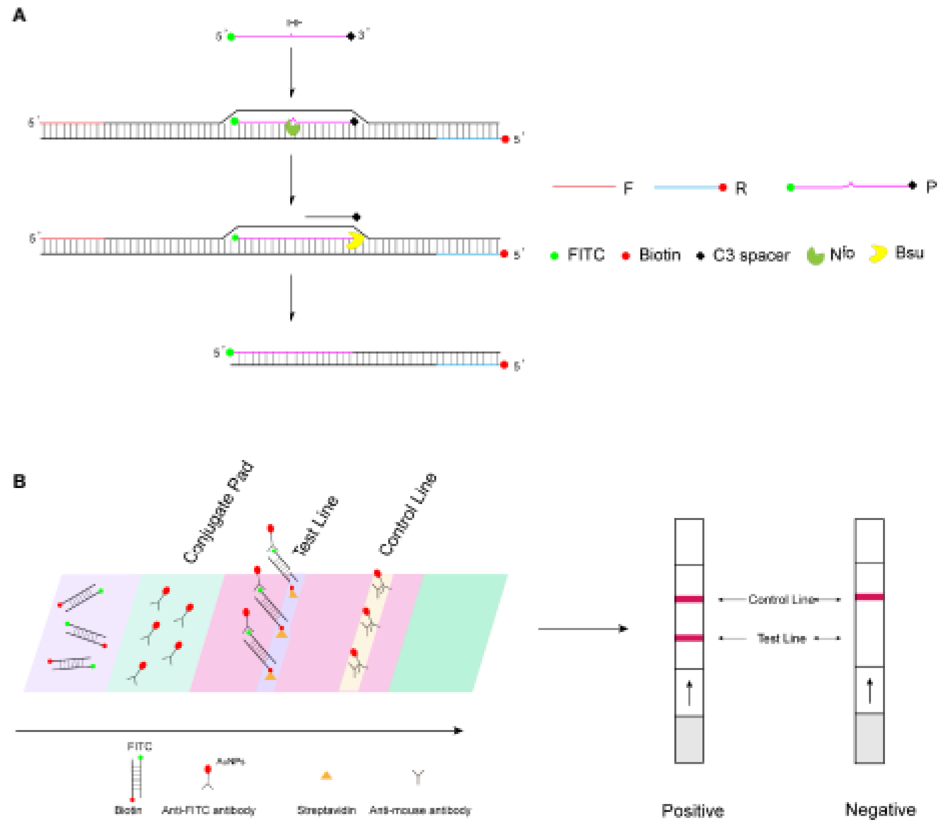
Table 4. Efficacy of rapid, field-deployable SHERLOCK assay

Method	Reaction Time min/C_T	Temperature (°C)	Sensitivity (Copies/reaction)	Reliability	Specificity	Lab dependency	Complexity
SHERLOCK assay	7	39	1	Highly correlated with qPCR assay (r= 0.93)	100%	no	Primers for RPA, guide RNA for CRISPR design complex
OIE recommended qPCR	30	60-94	12	100%	100%	yes	Primers available
Lateral flow strip			10 synthetic DNA	NA	100%	no	Need to design probe

Source: Sullivan *et al.*, 2019

3.3 Recombinase polymerase amplification combined with lateral flow test strip technology (RPA-LFS)

Polymerase chain reaction and quantitative fluorescent PCR, rely on laboratory equipment and are not suitable for field testing. So as a solution combination of Recombinase Polymerase Amplification (RPA) and Lateral Flow Test Strip Technology was adopted. This method targets the entire genome and designs primers and probes accordingly. Within 30 minutes and at 37° C.the detection can be completed (Zhang *et al.*, 2022). It does not rely on laboratory equipment and has broad application prospect in remote areas.



Source: Zhang *et al.*, 2022

Figure 8. RPA-LFS based detection mechanism diagram. (A) Principles of RPA amplification, (B) Visualization of lateral flow test strips. [F= Forward primer; R= Reverse primer; P= Probe]

Therefore, specific primers for RPA was designed combined with probes to ease colorimetric detection by LFS (Fig. 8A). The 5′ end of the probe was labeled with a fluorescent group (fluorescein isothiocyanate (FITC)) and the 5′ end of the reverse primer was labeled with biotin, with the aim of amplifying a target product with both a fluorescent group (FITC) and biotin. For the LFS assay, the control line on the strip is labeled with the anti-mouse antibody and the test line with streptavidin. The FITC end of the double-labeled RPA amplification product binds to the AuNPs of the bonding pad (wrapped by the anti FITC antibody), and then the biotin end binds to the streptavidin on the test line, showing a red positive band, while the AuNPs not bound to the amplification product bind to the anti-mouse antibody on the control line, showing a red color (Fig. 8B).

So, the fluorescence in probe (FITC) is used to visualize the target DNA of pathogen in the Test line by letting Anti-FITC antibody to get attached to the target DNA and giving red color to it through the AuNPs present in Anti-FITC antibody. On the other hand, The

fluorescence (Biotin) placed in the 5’ end of the reverse primer stay attached with the target amplified DNA and let the amplified DNA to get attached to the test line through binding with Streptavidin which is present in the test line already.

So, probe helps to visualize the pathogen’s presence and primer helps to visualize the pathogen’s presence on a particular line (test line). In this particular assay, the techniques used in the three basic steps of pathogen detection are listed in Table 5.

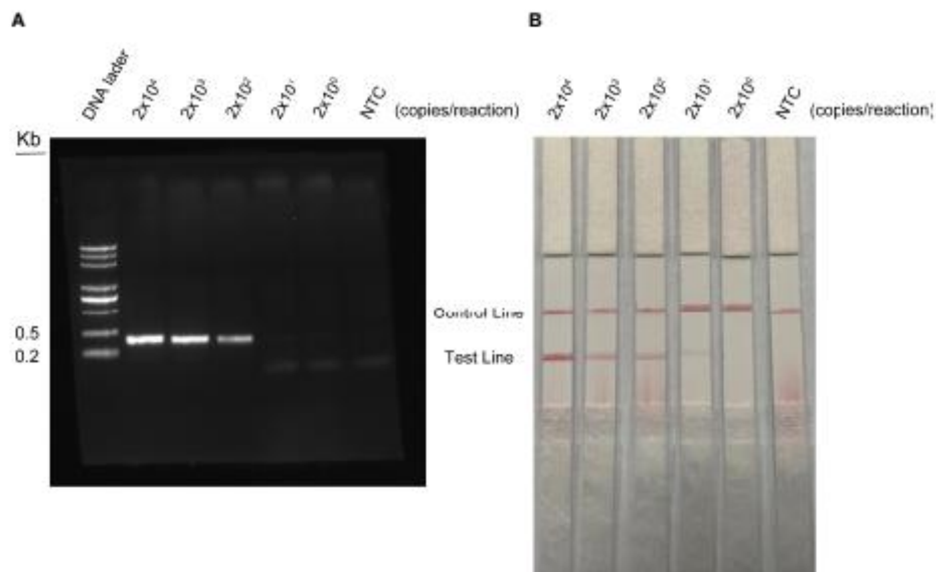
Table 5: Techniques used in RPA-LFS assay

DNA Extraction	**Amplification assay**	**Confirmation assay**
Commercial kit- Tiangen Biotech co. LTD, Beijing, China	Recombinase Polymerase Amplification using Twist Amp Liquid DNA Amplification kit	Lateral Flow Test Strip

Source: Zhang *et al.*, 2022

3.3.1 Sensitivity of RPA-LFS assay

To test the sensitivity of the RPA-LFS assay, standard plasmid of WSSV genomic DNA was diluted from 2×10^4 to 2×10^0copies/ml, agarose gel electrophoresis could detect 200 copies/ reaction (Fig.9 A), whereas the RPA-LFS could detect 20 copies/ reaction (Fig.9 B). So RPA-LFS is more sensitive assay than using agarose gel electrophoresis with RPA.



Source: Zhang *et al.*, 2022

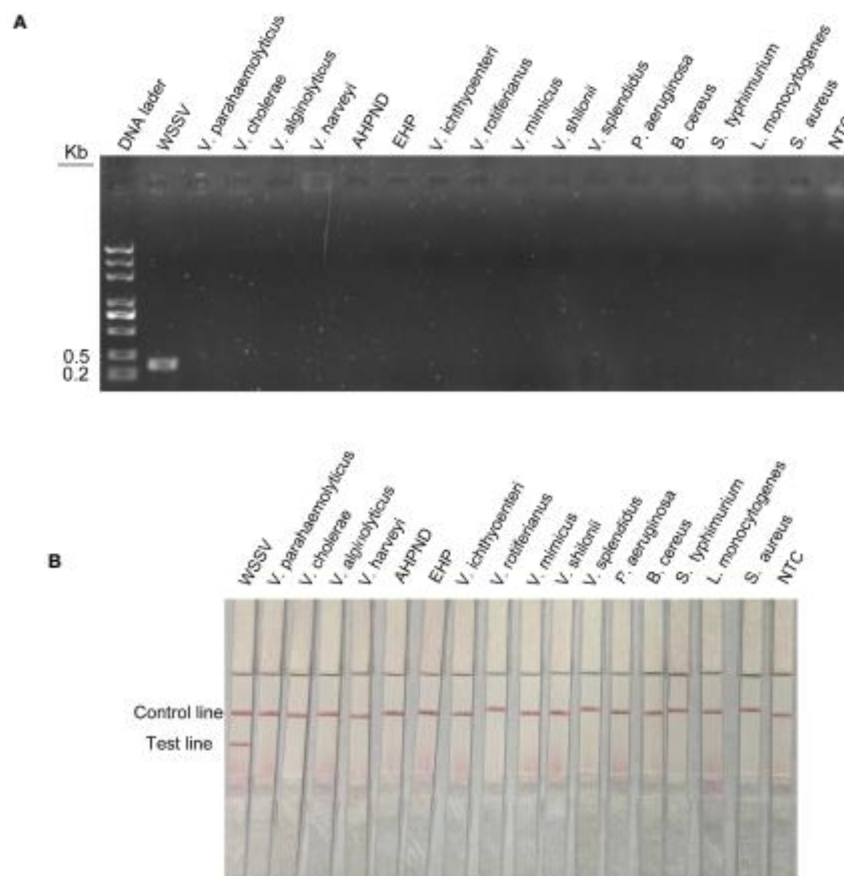
Figure 9. Sensitivity of the RPA-LFS assay for WSSV

[One microliter of WSSV (from 2×10^4 to 2×10^0copies/ml)was used as template and reacted at 37℃ for 30 min. (A) The result of the agarose gel electrophoresis. (B) The result of the LFS analysis. NTC = template-free negative control.]

Although the RPA-LFS technology used in this study is not as sensitive as SHERLOCK and real-time RPA technology, it avoids the expensive detection of SHERLOCK technology and the equipment dependence of real-time RPA detection.

3.3.2 Specificity and reliability of RPA-LFS assay

Specificity of RPA-LFS assay for WSSV was measured by using common shrimp pathogens as detection templates including *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Salmonella typhimurium* etc.



Source: Zhang *et al.*, 2022

Figure 10. Specificity of RPA-LFS assay for WSSV

[From the standard plasmid, 1 µl of 2×10^9 copies/ml was used as the template and reacted for 30 min at 37°C. The remaining templates are the common shrimp disease pathogens. NTC was a template-free negative control.]

The agarose gel electrophoresis could specifically detect WSSV and showed negative results for all the other pathogens (Figure 10 A). Similarly RPA-LFS assay was positive for WSSV and negative for all other pathogens (Figure 10 B). So the specificity of RPA-LFS assay is 100%.

3.3.3 Reliability of RPA-LFS assay

Table 6. Reliability assessment of RPA-LFS assay in detection of WSSV in clinical samples

Sample number	**RPA-LFS Assay**	**qPCR assay**		**Sample number**	**RPA-LFS Assay**	**qPCR assay**	
		Result	**Ct (n=3)**			**Result**	**Ct (n=3)**
No. 1	-	-	-	No. 26	-	-	-
No. 2	-	-	-	No. 27	-	-	-
No. 3	-	-	-	No. 28	-	-	-
No. 4	-	-	-	No. 29	-	-	-
No. 5	-	-	-	No. 30	-	-	-
No. 6	+	+	12.84	No. 31	-	-	-
No. 7	-	-	-	No. 32	-	-	-
No. 8	-	-	-	No. 33	-	-	-
No. 9	-	-	-	No. 34	-	-	-
No. 10	-	-	-	No. 35	-	-	-
No. 11	-	-	-	No. 36	-	-	-
No. 12	-	-	-	No. 37	-	-	-
No. 13	+	+	32.51	No. 38	-	-	-
No. 14	-	-	-	No. 39	-	-	-
No. 15	-	-	-	No. 40	-	-	-
No. 16	-	-	-	No. 41	-	-	-
No. 17	-	-	-	No. 42	-	-	-
No. 18	-	-	-	No. 43	-	-	-
No. 19	+	+	16.00	No. 44	-	-	-
No. 20	-	-	-	No. 45	-	-	-
No. 21	-	-	-	No. 46	+	+	13.73
No. 22	+	+	31.65	No. 47	-	-	-
No. 23	-	-	-	No. 48	-	-	-
No. 24	-	-	-	No. 49	-	-	-
No. 25	-	-	-	No. 50	-	-	-

Source: Zhang *et al.*, 2022

Table 6. (Continued)

Sample number	RPA-LFS assay	qPCR assay		Sample number	RPA-LFS assay	qPCR assay	
		Result	Ct (n=3)			Result	Ct (n=3)
No. 51	+	+	32.63	No. 66	-	-	-
No. 52	-	-	-	No. 67	-	-	-
No. 53	-	-	-	No. 68	-	-	-
No. 54	-	-	-	No. 69	-	-	-
No. 55	-	-	-	No. 70	-	-	-
No. 56	-	-	-	No. 71	-	-	-
No. 57	-	-	-	No. 72	-	-	-
No. 58	+	+	33.83	No. 73	-	-	-
No. 59	-	-	-	No. 74	-	-	-
No. 60	-	-	-	No. 75	-	-	-
No. 61	-	-	-	No. 76	+	+	13.66
No. 62	-	-	-	No. 77	-	-	-
No. 63	-	-	-	No. 78	-	-	-
No. 64	-	-	-	No. 79	-	-	-
No. 65	-	-	-	No. 80	-	-	-

The RPA-LFS detection technology can detect 8 positives in the 100 clinical samples, and the consistency rate with the qPCR detection results is 100%. So, it can be said that RPA-LFS assay is a highly sensitive method.

3.3.4 The efficacy of RPA-LFS assay as a rapid, on-field detection assay is described by comparing with ideal qPCR method based on different parameters in Table 7.

Table 7. Efficacy of RPA-LFS assay

Method	Reacti-on Time min/CT	Temperature (℃)	Sensitivity (Copies/reaction)	Reliability	Specificity	Lab dependency	Complexity
RPA-LFS assay	30	37	20	100%	100%	no	Primers, probe deign complex
qPCR	30	60-94	12	95%	100%	yes	Primers available

Source: Zhang *et al.*, 2022

3.4 Comparison of the efficacy of different rapid detection methods used for WSSV detection.

Table 8. Comparison of the efficacy of different rapid detection methods used for WSSV detection.

Method	Reacti-on Time min/C_T	Temperature (℃)	Sensitivity (Copies/reaction)	Reliability	Specificity	Lab dependency	Complexity
qPCR (Xia *et al.*, 2014)	30	60-94	12	95%	100%	yes	Primers available
Real-time RPA (Xia *et al.*, 2014)	7	39	5	87.5 %	100%	no	Primers deign complex
SHERLOCK assay (Sullivan *et al.*, 2019)	7	39	1	Highly correlated with qPCR assay (r= 0.93)	100%	no	Primers for RPA, guide RNA for CRISPR design complex
RPA-LFS assay (Zhang *et al.*, 2022)	30	37	20	100%	100%	no	Primers, probe deign complex

3.5 Feasibility and limitations of detection methods for rapid detection of fish pathogen

From the data presented in the Table 8, qPCR, real-time RPA and RPA-LFS assay all methods used extracted DNA by commercial DNA extraction kit which needs extra time. So the actual time needed for the detection of pathogen is not limited within the reaction time. These 3 assays have less sensitivity and thus there is a chance of disease outbreak if ignored due to failing in detection.

On the other hand CRISPR based SHERLOCK method is the most sensitive one and gives rapid result as this assay was combined with Paper matrix based nucleic acid extraction and from DNA extraction to the final detection the whole assay can be performed within 1 hour at 39℃. But the designing of primers, reporter RNA, guide RNA for SHERLOCK method is complex and a costly process. So the final cost for this assay might be high for farmers and a little bit complex to understand by them. With proper training if the farmers get familiar with this assay and biotechnologist gets enough funding to design these primers, reporters and guide RNA then this method is feasible as a rapid detection method for fish pathogen detection.

CHAPTER IV

CONCLUSION

On the basis of the findings of this review paper, the following conclusions are drawn-

Recently few methods are reported that are developed to detect fish pathogens rapidly. In these recent advancements, manual DNA extraction or Kit dependent DNA extraction has been replaced with paper matrix based nucleic acid extraction which is not lab dependent. Real-time RPA, CRISPR-based sensitive high efficiency reporter unlocking (SHERLOCK) assay and RPA-LFS assay were adapted for amplification of DNA of fish pathogen in 2014, 2019 and 2022, respectively that overcome the issues of lab dependent assays. Lateral flow test strips made the detection assay easily visible, possible to be performed in the fish farm also.

On the basis of sensitivity, specificity, reliability, time needed for the assay, field deployability the efficacy of the real-time RPA, CRISPR- based SHERLOCK, and RPA-LFS was compared. The SHERLOCK assay have the sensitivity of single copy detection within 7 min and from DNA extraction to pathogen detection the whole assay takes only 1 hour and possible to conduct in the pond side thus proved to be the most efficient rapid detection assay.

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