Progression of Loop-Mediated Isothermal Amplification (LAMP) assay for detection of *Sitophilus oryzae* in food samples

TRUPTI N. BARBUDDHE
University Department of Chemical Technology (UDCT), Sant Gadge Baba Amravati University Amravati (SGBAU), Amravati, Maharashtra, India

Abstract- ***Post-harvest crop losses, due to insects and microbes, accrue significant economic waste in both developed and developing countries. The rice weevil (Sitophilus oryzae) is a stored product pest which attacks several crops, including wheat, rice, and maize. S. oryzae is able to develop on a wide range of cereals and also on processed cereal products such as pasta. A few strains of S. oryzae have been found which can develop on grain legumes peas, lentils and green or black grams are the pulses most often attacked by these strains. The infestation of stored product insects leads to the qualitative and quantitative deterioration of the commodity as they feed on them. The stored product may contain either adults, larvae, eggs or dead insect parts. Hence the aim of this work is to develop a isothermal amplification techniques to detect the eggs, larvae, adult insect and their remnants in different food matrices using specific primers. Primers were designed for 'internal transcribed spacer 2' genes of Sitophilus and LAMP conditions were optimized for the detection of Sitophilus oryzae. The Sitophilus oryzae contaminated food matrices were checked for matrix interference in LAMP analysis.***

Index Terms- ***Sitophilus oryzae, LAMP, stored product insect, food matrices***

I. INTRODUCTION

It has been evaluated that pests devour about 6.5% of total stored grains in India [1]. Even though ultramodern storage facilities are available, farmers in rural India due to their ignorance and lack of extension facilities in the hinterland, still depend upon traditional techniques of storage of food grains, thereby making their grains prone to the infestations of pests during storage [2]. Storage grain losses of major cereal crops can be attributed primarily to attack by rice insect pests, diseases, and rodents [3]. It is generally believed that half of the storage losses are usually caused by insects [4]. Of these pests, *Sitophilus oryzae L*. is the most cosmopolitan in nature, and causes severe losses in rice, maize, barley, wheat, and other crops [5-7]. Whereas the hot, humid climate of Southeast Asia is quite worthy for rice cultivation, it is equally worthy for rapid stored-product insect development which can result in explosive outbreaks, potentially causing devastating post-harvest rice grain damage. The rice weevil, *Sitophilus oryzae L*), is one of the most destructive pests of stored cereals worldwide. Thes susceptibility can be decreased by innovating a technique for early detection and targeting of insect infestations in stored triticale. Early noticing of rice weevil can be difficult because the larvae feed secret inside the kernels and there are no visible superficial indicators of damage until the adult come out. Nonvisual methods to detect insects inside grain kernels include acoustic sensing, X-ray, nuclear magnetic resonance, and visible and infrared spectroscopy [8-13]. Although there have been many studies for detection of various insect infestations in wheat and other cereals, they suffer from drawbacks. Considering these things, it is imperative to develop faster, sensitive, effective methods of detection of storage insect pests, and this is of paramount importance. PCR has been reported as a method of detection but PCR suffers from many drawbacks. Hence, we attempted to develop an isothermal amplification method using specific primers for the detection of *Sitophilus oryzae L* in few food matrices.

II. MATERIALS AND METHODS

2.1 Food Samples

Few food matrices were obtained from local market. Wheat used for growth of *S. oryzae* was conditioned and used according to Kalpana and Sumithra Devi

[14]. All buffers and chemicals used were purchased from standard chemical companies and were of analytical grade.

2.2 Isolation of insect Genomic DNA

Seven days old *S. oryzae* growing on wheat were used for DNA isolation. 0.1g insects were taken and DNA was isolated by phenol-chloroform method (method according to Sambrook et al. [15].

2.3 Primers

Two sets of primers were designed by using Primer 4 software. The primers of LAMP were got synthesized from a commercial company (Sigma Aldrich, Bangalore, India).

2.4 Loop-mediated Isothermal Amplification Assay

The LAMP reaction was conducted, following the methods described by Notomi et al. [16] and Nagamine et al. [17], with slight modifications. The LAMP assay was carried out in a 25 µL reaction mixture containing 1.6 mmol/L of each inner primer (forward inner primer (FIP) and backward inner primer (BIP)), 0.2 mmol/L of each outer primer (F3 primer and B3 primer), 0.8 mmol/L of each loop primer (both loop forward and loop backward or either individually), 1.4 mmol/L of deoxynucleotide triphosphate (dNTP), 0.5 mol/L betaine (Sigma, St. Louis, MO, USA), 20 mmol/L Tris-HCl (pH 8.8), 10 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, 8 mmol/L $MgSO_4$, 0.2% Tween-20, 8U/mL of the *Bst* DNA polymerase large fragment (New England Bio labs, Beverly, MA, USA) and 2 µL of the template DNA. The reaction mixture was incubated at 65˚C for 60 min and then heated at 80˚C for 2 min to terminate the reaction. LAMP products were subjected to electrophoresis on a 2% agarose gel, visualized under UV light after ethidium bromide staining and documented in Gel Documentation System (Cleaver Scientific Ltd, Rugby, Warwickshire, U.K.). For real-time monitoring of the LAMP assay, the reaction mixture after incubation at 65˚C for 60 min was sequentially treated with 1 mL of 1:10 SYBR Green I (Sigma).

2.5 Analysis of food samples

Different food samples were spiked with 10 insects (*S. oryzae* adults) per gram and DNA was isolated as given above.

III. RESULTS AND DISCUSSION

3.1 LAMP primers

Two sets of LAMP primers were designed using Primer 4 software. The primers were designed for ‘internal transcribed spacer 2’ gene. The primers are given in (Table 1).

Table 1: Oligonucleotide sequences of LAMP primers

Primer set	Oligo name	Oligonucleotide Sequence 5’ to 3’
I	FIP	5’ACTCCGAAAGTTTCTTTTTTTCCTCGTTTACATTTTAATTCTCCCAGG
	BIP	5’TCGCAATTGGTTTATTAGGATTCGTGTATCAACGTCTATTCCTACTG
	F3	5’TTCTTCGGACACCCAGAA
	B3	5’GCTGATGTAAAGTATGCTCGT
	F1c	5’ACTCCGAAAGTTTCTTTTTTTCCTC
	B1c	5’TCGCAATTGGTTTATTAGGATTCGT
	F2	5’GTTTACATTTTAATTCTCCCAGG
	B2	5’GTATCAACGTCTATTCCTACTG
	FIP	5’CGAGCCAAGTGATCCACCGTTTTTCGAGGCAAACTC
	BIP	5’AGCGCATCGATGAAGAACGCATTCATGTGTCCTGCAGTTCA
	F3	5’CGACTACGACGAAGTGAGC
	B3	5’ATCCGAGGACCGCAATGT
	F1c	5’CGAGCCAAGTGATCCACCGTTT
	B1c	5’AGCGCATCGATGAAGAACGCA

II	F2	5'TTCGAGGCAAACTCGTCAAT
	B2	5'GTATCAACGTCTATTCCTACTG

3.2 Influence of reaction temperature on LAMP reaction

LAMP reaction was carried out at various temperatures 25˚C, 37˚C and 65˚C, for 60 min. With both primers, low amplification of product was found at 25˚C and 37˚C, but products were found at reaction temperature of 65˚C. Therefore, optimized LAMP conditions were maintained at 65˚C in further studies.

3.3 Influence of reaction time on LAMP reaction

LAMP reaction was carried out at various incubation periods, i.e., 10min, 20min, 30min, 40min, 50min, 60min, 70min, 80min, 90min, 100min, 120min, 180min, 240min and 300min at 65˚C. With primer 1, no amplification of product was found in the reaction time of 10min. The amplification increased with time. Maximum fluorescence was observed by 60 min and further increase was very negligible (Figure 1). With primer 2, maximum fluorescence was observed by 60 min and further increase was low. Visible green fluorescence also could be clearly observed by 60min, optimized LAMP conditions were maintained at 65˚C for 60 min in further studies.

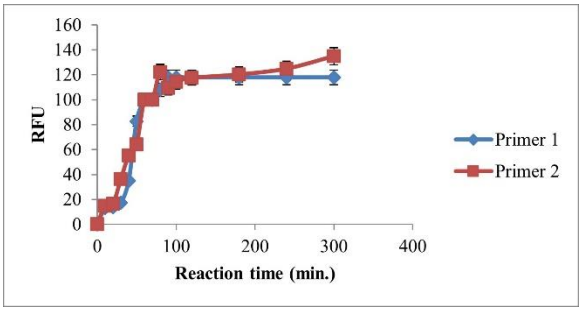

Figure 1: LAMP reaction as a function of time in the detection of *S.oryzae* (L)

3.4 Effect of DNA concentration on LAMP reaction

Insect genomic DNA concentrations from 60ng to 1200ng were studied for fixing the DNA concentration required for LAMP reaction. Visible green fluorescence of SYBR Green was low at 60ng (5insects) DNA levels. Visible green fluorescence increased with increase in DNA concentration and was maximum at 240ng (Figure 2) of DNA (20 insects). Both the primers exhibited the same trend.

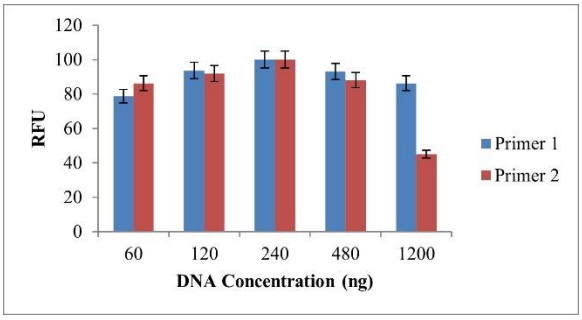

Figure 2: LAMP reaction as a function of DNA concentration

3.5 Real time analysis

The amplification was recorded as change in the colour of SYBR Green from orange to fluorescent green with time 10 min – 60 min (Figure 3).

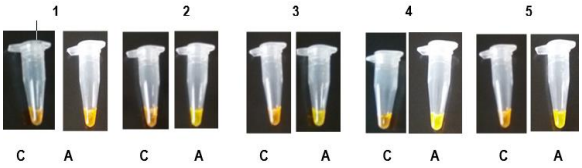

Figure 3: Real time analysis

1- Amplification recorded at 10 min; 2- Amplification recorded at 20 min; 3- Amplification recorded at 30 min; 4- Amplification recorded at 45min; 5- Amplification recorded at 60 min; C- Control sample; A- Amplified samples.

3.6 Different stages of growth VS LAMP amplification

The detection of S.oryzae could be done at all stages of growth. Insect spiked wheat and rice samples of different ages from 0 day to 30 days were attempted. Rice and wheat were spiked with *S.oryzae*. For egg stage 6-7 day old spiked grain was taken. For larval stage 12 – 15 day old grain was collected. 25-30 day old insect was chosen for pupal stage. Adults were collected after emergence [18]. DNA was isolated and LAMP assay was done as mentioned above.

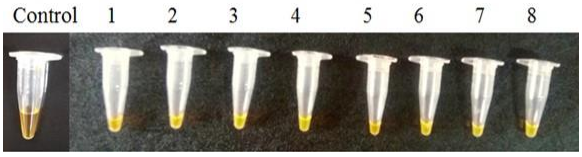

Figure 4: LAMP amplification of different stages of growth on wheat matrix

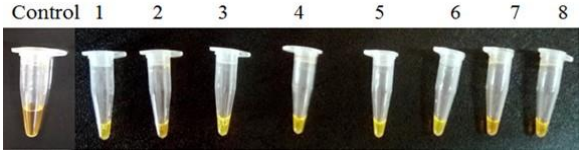

Figure 5: LAMP amplification of different stages of growth on rice matrix

Tubes 1-4: Primer I; Tubes 5-6: Primer II
Tube 1- egg stage; Tube 2- larval stage; Tube 3- pupal stage; Tube 4- adult stage; Tube 5- egg stage; Tube 6- larval stage; Tube 7- pupal stage; Tube 8- adult stage.

The presence of insects could be detected at all stages of growth from egg to adult stage in both wheat (Figure 4) and rice (Figure 5) matrix.

3.7 LAMP reaction is artificially infested food samples

Wheat and wheat base products, rice and rice base products, and other grains black gram and bengal gram were used as food matrices. *S. oryzae* (L) was spiked in to these food matrices and LAMP assay was done using the DNA isolated in presence of Food matrix. LAMP reaction could detect *S. oryzae* (L) in all matrices used except one type of biscuits where, there was maximum matrix interference (Table 2).

Table 2: Influence of food matrix in the detection of *S.oryzae*

Commodity	Type	Amplification	
		Primer I	Primer II
Wheat	Wheat grains	++	++
	Wheat flour	++	++
	Biscuits 1	Matrix interference	Matrix interference
	Biscuits 2	++	++
	Biscuits 3	++	++
	Biscuits 4	++	+
	Biscuits 5	+	+
Rice	Rice grains	++	++
	Rice flour	++	++
	rice noodles	++	++
	Rice flakes	+	+
Black gram	Grains	+	+
Bengal gram	Grains	+	+

+ low amplification, ++ good visible amplification

IV. DISCUSSION

The rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), is one of the foremost damaging pest of stored cereals around the world. It is classed as a primary pest, cosmopolitan in nature and is known to infest sound cereal seeds [19] and causes severe loss in rice, maize, barley and wheat [5-7]. Reports about its occurrence on legumes are scanty. In India, the pest was recorded for the first time to feed on red gram at Coimbatore [20]. Techniques such as Computed tomography [21], automatic bioacoustic recognition [22], uses of visible and near-infrared spectral signatures [23], Nonvisual methods to detect insects inside grain kernels include acoustic sensing, X-ray, nuclear magnetic resonance, and visible and infrared spectroscopy [8-13] have been adopted. All though many of these techniques have been reported, they suffer from their own inherent drawbacks. Considering these, faster, cost effective and simple methods of detecting these insects at early stages is of paramount importance. PCR method for detection of *Sitophilus oryzae* (L.) has been developed [24, 25]. But PCR technique has its own disadvantages such as requirement of PCR machine and post amplification analysis by agarose gel electrophoresis followed by documentation of the amplification. We have developed isothermal DNA amplification technique for detection of *Sitophilus oryzae* (L.), wherein the

nucleic acid amplification can be done at one temperature, i.e. the amplification can be in a water bath. To our knowledge, this is the first report on LAMP based amplification detection of *Sitophilus oryzae* (L). LAMP is a novel approach to nucleic acid amplification that uses a single-temperature incubation, thereby obviating the need for expensive thermal cyclers. LAMP is based on autocycling strand displacement and the amplicons are stem-loop DNA structures with several inverted repeats of the target. It has been shown that LAMP exhibits less sensitivity to inhibitory substances present in the biological samples than PCR. The LAMP assay produces a large amount of amplified product, resulting in easier detection by visual inspection, by an increase in turbidity caused by generation of magnesium pyrophosphate or a color change after the addition of SYBR Green 1 dye. All samples that were positive by gel electrophoresis were also positive by visual detection of color change and *vice versa*. Color change of the solution has good correlations with the amount of DNA synthesized [26]. The present study describes the development of a novel technique involving one-step isothermal gene amplification assay for rapid detection and identification of *Sitophilus oryzae* (L.) in the food samples.

We have attempted to study matrix effect in the LAMP based detection of *Sitophilus oryzae* (L.). We designed two sets of primers and used them in this study. Both the primers showed good amplification. We could detect *Sitophilus oryzae* (L.) present in food matrices.

Thus, LAMP method was found to be very useful over PCR, as it is simple, requires only water bath and can be accomplished under isothermal condition in 1 h or less. LAMP method has been used for the detection of many bacterial specie [27]. LAMP of DNA is a novel technique. Its high specificity, cost-effectiveness and simple procedure, especially suited to fit into the daily work requirement of food quality control laboratories, made the LAMP a promising tool for developing countries, such as India, where there is concern with respect to their potential for serious food-infestation and food loss, in order to heighten consumers' protection and to alleviate infestation to considerable extent.

REFERENCES

[1] Raju P. 1984. The staggering storage losses - causes and extent. Pesticides 1984 Vol. 18 No.1pp. 35-37.

[2] Aslan, I.; H. Ozbek; S, Kordali; O, Calmasur and A, Cakir. 2004. Toxicity of essential oil vapours obtained from Pistacia spp. to the granary weevil, Sitophilus granarius (L.) (Coleoptera: Curculionidae). Journal of Plant Diseases and Protection 111: 400–407.

[3] Rana, P. N. and G. K. KC. 1977. Report on the studies of different types of storage structures in Nepal. Nepalese Journal of Agriculture 12: 203-215.

[4] FAO. 1968. Rice grain of life. International Rice Year 1966: Freedom from hunger. World Food Problems No. 6. Food and Agriculture Organization of the United Nations, Rome, Italy, 65p.

[5] Bhatia, S. K., Singh, V. S. and Bansal, M. G. (1975). Varietal resistance in barley grain to laboratory infestation of rice weevil and lesser grain borer. Bulletin of Grain Technology 13 (2): 69-72.

[6] Singh, V. S., Bhatia, S. K. and Murthy, B. N. (1980). Effect of hull on the resistance of barley varieties to the rice weevil *Sitophilus oryzae* L. infestation. Indian Journal of Entomology 42 (4): 576-581.

[7] Neupane, F. P. (1995). Agricultural Entomology in Nepal. Review of Agricultural Entomology 83 (12): 1291-1304.

[8] Mankin, R., Hagstrum, D., 2011. Acoustic monitoring of insects. In: Hagstrum, T.W.P.D.W., Cuperus, G. (Eds.), Stored Product Protection, Publication. Kansas State Univ. Press, Manhattan, KS, 1566eS222.

[9] Singh, C.B., Jayas, D.S., Paliwal, J., White, N.D.G., 2009. Detection of insect-damaged wheat kernels using near-infrared hyperspectral imaging. J. Stored Prod. Res.45, 151e158.

[10] Newey, P.S., Robson, S.K.A., Crozier, R.H., 2008. Near-infrared spectroscopy identifies the colony and nest of origin of weaver ants, Oecophylla smaragdina. Insectes Sociaux 55, 171e175.

[11] Neethirajan, S., Karunakaran, C., Jayas, D.S., White, N.D.G., 2007. Detection techniques for stored product insects in grain. Food control. 18, 157e162.

[12] Tigabu, M., Oden, P.C., Shen, T.Y., 2004. Application of near-infrared spectroscopy for the detection of internal insect infestation in Picea abies seed lots. Can. J. For. Res. 34, 76e84.

[13] Dowell, F.E., Throne, J.E., Baker, J.E., 1998. Automated nondestructive detection of internal insect infestation of wheat kernels by using near-infrared reflectance spectroscopy. J. Econ. Entomology. 91, 899e904.

[14] Kalpana C. Devi and Sumithra S. Devi.,2013. Insecticidal and oviposition deterrent properties of some spices against coleopteran beetle, Sitophilus oryzae. J Food Sci Technol. 50(3): 600–604.

[15] Sambrook, J; Frisch, E.F; & Maniatis, T. (1989). *Molecular Cloning: A laboratory manuel. Cold Spring Harbour*. 2nd edn. New York: Cold Spring Harbor Laboratory Press.

[16] Notomi, T.,Okayama, H.,Masubuchi, H., Yonekawa, T.,Watanabe, K. and Amin, N. 2000. Loop-mediated isothermal amplification of DNA.Nucleic Acids Res. 28, e63.

[17] Nagamine, K., Hase, T. and Notomi, T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers.Mol. Cell. Probes. 16, 223–229.

[18] Singh, P.K.B. 2017. Study on the life cycle of Sitophilus oryzae on rice cultivar pusa 2-21 in laboratory condition. International Journal of Education & Applied Sciences Research.4, 37-42.

[19] Hill, D. S. (1990). Pests of Stored Products and Their Control, Belhaven press, London

[20] Vijay S, K. Bhuvaneswari and G. Gajendran, 2015, Assessment of grain damage and weight loss caused by *Sitophilus oryzae* (L.) feeding on split pulses, Agric. Sci. Digest., 35 (2) 2015: 111-115.

[21] Toews MD, Pearson TC, Campbell JF. Imaging and automated detection of *Sitophilus oryzae* (Coleoptera: Curculionidae) pupae in hard red winter wheat, J Econ Entomol. 2006 Apr;99(2):583-92.

[22] Potamitis I, Ganchev T, Kontodimas D. On automatic bioacoustic detection of pests: the cases of *Rhynchophorus ferrugineus* and *Sitophilus oryzae*. J Econ Entomol. 2009 Aug;102(4):1681-90.

[23] M.K. Khedher Agha, W.S. Lee, C. Wang, R.W. Mankin, A.R. Blount, R.A. Bucklin, N. Bliznyuk, Detection and prediction of *Sitophilus oryzae* infestations in triticale *via* visible and near-infrared spectral signatures, Journal of Stored Products Research 72 (2017) 1e10.

[24] Obrepalska-Steplowska, A. Nowaczyk,K. Holysz, Gawlak, M.M. Nawrot, J Molecular techniques for detection of granary weevil (*Sitophilus granarius* L.) in wheat and flour, Food Additives and Contaminants 2008, 1-31.

[25] Kaneko, H., Kawana, T., Fukushima, E. and Suzutani, T. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J Biochem Biophys Methods (2007) 70, 499–501.

[26] Ji J., Xie., Q. M., Chen., C. Y., Bai. S. W., Zou. L. S., Zuo. K. j., Cao. Y. C., Xue. C. Y., Ma. j. Y., and Bi. Y. Z. Molecular detection of Muscovy duck parvovirus by loop-mediated isothermal amplification assay. Poultry Science (2010) 89 :477–483.

[27] Sowmya N, Thakur M S and Manonmani H K, Detection of enterotoxigenic Staphylococci by loop-mediated isothermal amplification method, Journal of Food Safety 32 (2012) 59–65.