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Standardisation of loop mediated isothermal amplification assay for rapid detection of New Duck disease

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Riemerella anatipestifer, the causative organism of the epidemic New Duck disease, is difficult to be diagnosed due to its morphologic similarity with *Pasteurella* spp as well as its sharing of clinical signs with *Escherichia coli* infections and salmonellosis. The ducks in the coastal areas of Kerala, India get affected with New Duck disease every year especially after the onset of monsoons resulting in huge morbidity and eventually imparting heavy economic loss upon duck farmers. Hence prompt control measures against New Duck disease are needed in order to curtail the spread of the outbreak (Pala and Radhakrishnan 2014).The outer membrane protein A (*ompA*) gene of *R. anatipestifer*, has role in the virulence of the organism and is a good candidate for developing diagnostic and control strategies for New Duck disease (Somu *et al.* 2016).

Diagnostic tests based on nucleic acid amplification like polymerase chain reaction (PCR) assays often act as rapid and precise diagnostic tools (Rodriguez 1997, Yang and Rothman 2004, Parida *et al.* 2008). Despite its advantages, the need for costly thermal cycler and post amplification processing in PCR hinder its use as routine diagnostic tool in low resource setting laboratories (Rekha *et al.* 2014). Many isothermal nucleic acid amplification techniques such as Transcription Mediated Amplification, Strand Displacement Amplification, Rolling Circle Amplification, Helicase Dependent Amplification etc. also serve as diagnostic tools; of which Loop-mediated isothermal amplification (LAMP) assay is very widely used (Dhama *et al*. 2014). The major advantage associated with LAMP technique is that nucleic acid amplification can be carried out in less time as compared to PCR without compromising the sensitivity and specificity. Besides, result can be interpreted based on visual detection which is not possible with PCR. Developments in LAMP assays are highly pertinent in resource limited laboratories of developing countries (Geojith *et al.* 2011). Hence, the present study was designed to standardize a LAMP assay targeting the *ompA* gene of *R. anatipestifer* for the field level diagnosis of New Duck disease in Kerala.

The well characterized isolates of *R. anatipestifer* maintained in the Department of Veterinary Biochemistry of the institute viz. KML-1, KML-2 and KML-3 were used for the study. Ready to use sterile sheep blood agar plates procured from Himedia Laboratories Private Limited, Mumbai were used for revival and subculture of the isolates. The bacterial isolates were identified based on morphology, cultural characteristics, growth on MacConkey agar, haemolysis on blood agar and biochemical tests like catalase and oxidase, indole production, gelatin liquefaction and ornithine decarboxylase activity. Colonies were collected from blood agar and suspended in 500 µl of distilled water and boiled at 100°C for 15 min. After centrifugation at 17,000 × g for 10 min, the supernatant was collected and used as the source of DNA for the assay. Final confirmation of the obtained isolates was done by amplifying a partial region (665 bp) of 16S rRNA gene as per protocol and primers described by Pala *et al.* (2013). Those isolates confirmed as *R. anatipestifer* were used for further analysis.

Four sets of primers (two inner and two outer) based on the conserved region of the *ompA* gene of *R. anatipestifer* (Zheng *et al*. 2011) were used in the study. These primers were used in a 25 µl reaction mixture containing 2 µl DNA template, 10 picomoles of each primer, 10 mmol/l each of dATP, dCTP, dGTP, and dTTP; 100 mmol/l $MgSO_4$ and 5M l betaine. After heating at 95ºC for 5 min and snap-chilling on ice, 8 U Bst DNA polymerase (New England BioLabs) was added. The reaction mixture was then incubated at 62ºC for 60 min and held for 2 min at 80ºC to terminate the reaction.The LAMP products generated were electrophoresed in 2% agarose for 1h. Visual inspection of the LAMP products was done by the naked eye under UV light after adding 1µl of SYBR green I dye (10000×concentrate in DMSO) (Invitrogen, USA) to the amplicons after completion of the reaction.

All the three isolates selected were amplified with 16S rRNA primers giving a 665 bp DNA product (data not shown) at an annealing temperature of 54°C confirming

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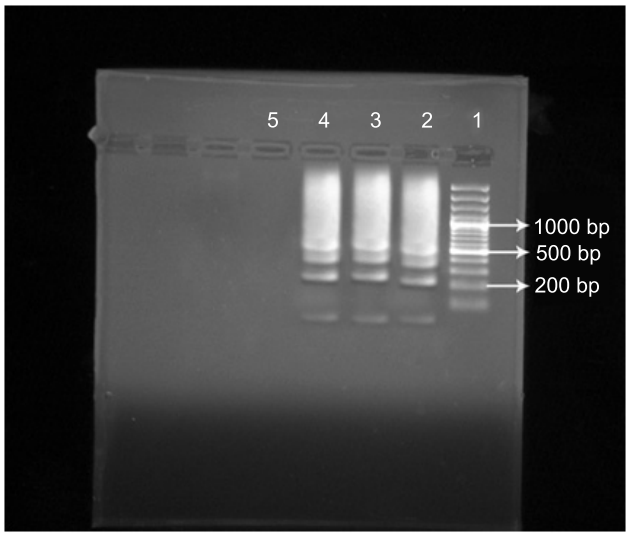

Fig. 1. LAMP amplified products of *R. anatipestifer.* Lane 1, 100bp ladder; lane 2, KML-1; lane 3, KML-2; lane 4, KML-3; lane 5, control.

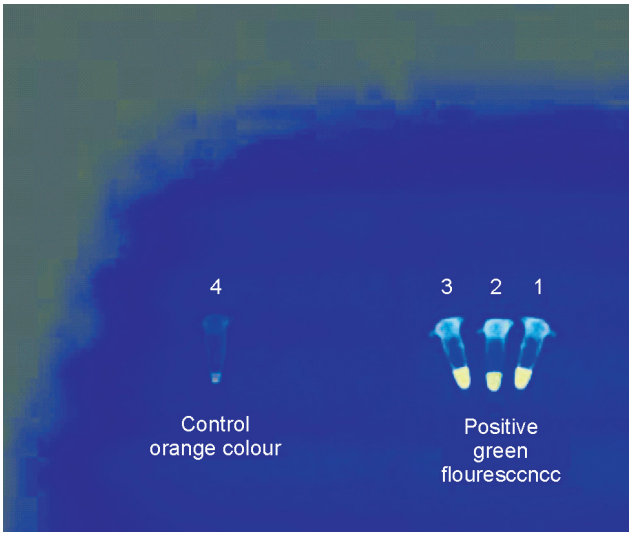

Fig. 2: Visual detection of LAMP amplified products under UV light. Tube 1, 2 and 3- Green fluorescence (positive for *R. anatipestifer*)*;* tube 4 - orange colour; negative control.

their identity as *R. anatipestifer*. All the three isolates were successfully amplified by the LAMP assay targeting *ompA* gene of *R. anatipestifer*. The amplicons obtained were confirmed by agarose gel electrophoresis which depicted a ladder pattern, i.e, varying sizes of bands, in all the three isolates (Fig. 1). The LAMP amplified products were also visualized by the naked eye directly under ultraviolet light following the addition of SYBR Green I to the reaction mixture, where a positive reaction was indicated by the formation light green fluorescence. The control (assay without DNA template) displayed an orange colour indicative of a negative reaction (Fig. 2). Similar findings were observed by Zheng *et al.* (2011) where LAMP assay targeting *ompA* gene gave positive results for eight reference strains, 149 isolated strains and 149 infected duck brains. Han *et al*. (2011) developed a LAMP assay targeting *GroEL* gene for rapid detection of *R. anatipestifer* which ascertained that LAMP assay is a very effective method to diagnose New Duck disease.

Loop-mediated isothermal amplification is a simple, cost-effective, and rapid method for the specific detection of genomic DNA that uses a set of four or six oligonucleotide primers with six or eight binding sites hybridizing specifically to different regions of a target gene, and a thermophilic DNA polymerase from *Geobacillus stearothermophilus* for DNA amplification (Han *et al.* 2011). This method has been applied successfully for the detection of many pathogens. Compared to PCR, LAMP has the advantages of reaction simplicity and detection sensitivity. The PCR-based methods are often cumbersome to adapt for routine clinical use especially in peripheral healthcare settings and private clinics. Moreover, the equipment required for conventional PCR and real-time PCR assays is relatively expensive, and these techniques are complex to perform in resource-limited laboratory settings in developing countries (Lee *et al*. 2015). Another benefit of the LAMP assay lies in the opportunity for detection of a positive reaction without the use of electrophoresis. The LAMP reaction product could be easily seen under ultraviolet light with addition of SYBR Green I into the LAMP reaction. These characteristics of the reaction make it an ideal field level diagnostic tool for New Duck disease.

SUMMARY

In the present study, a loop-mediated isothermal amplification assay targeting the *ompA* gene of *R. anatipestifer* was standardised using three freeze dried isolates of the organism. The reaction could be completed in a short time i.e. approximately an hour, as compared to PCR which requires about 3 h for completion. The whole isothermal reaction was performed in a simple water bath and the positive reaction could be detected with naked eye, thereby confirming that no expensive equipment is needed for the diagnosis of the pathogen effectively. This technique could be mastered by veterinarians and laboratory technicians very easily compared to PCR; thereby reducing the time lag in reaching a confirmative diagnosis. This assay could be carried out in a local laboratory without any special equipment and high edge facilities.Once properly standardized, the LAMP assay serves as a very simple, rapid and effective pen-side diagnostic technique. It has the potential to revolutionize the field of human and veterinary disease diagnosis, thereby aiding in adopting prompt control and therapeutic strategies.

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