DEVELOPMENT OF A COLORIMETRIC LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR DETECTION OF PATHOGENIC *Streptococcus* SPECIES IN AQUACULTURE

By

NUR MUHAMAD SYAHIR BIN ABDUL HABIB

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chairman : Nur Adeela binti Yasid, PhD
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Initially thought to be resistant to most bacterial infections, outbreaks in aquatic farms showed that tilapias are indeed susceptible to various diseases such as streptococcosis. Infections by *Streptococcus* spp. particularly *Streptococcus agalactiae* and *Streptococcus iniae* have been reported to cause major outbreaks affecting tilapia producers and farmers. Rapid and accurate detection of pathogens with a point-of-care (POC) application is compulsory to reduce the losses due to the disease. While the polymerase chain reaction (PCR) is a widely used conventional technique for DNA amplification in pathogen detection, it has some disadvantages such as slightly cost-inefficient, equipment complexity, necessitates precise temperature cycling and routine post-amplification analysis; and prone to false-positives results. Alternative technique such as the loop mediated isothermal amplification (LAMP) generally offers a simpler and more cost-effective alternative, specifically in terms of equipment requirements, simplicity of operation and reduced risk of false positives. These attributes make LAMP a promising choice for field-based testing and POC applications where rapid and reliable nucleic acid amplification is essential. Despite the advantages of LAMP in terms of simplicity, rapidity, and sensitivity, there is still a gap in the development of a specific LAMP assay tailored to accurately and reliably detect specific pathogens for enhanced disease management and surveillance. This project intends to address this gap by designing, optimising, and validating a novel and specific colorimetric detection tool using gold nanoparticles (AuNPs) probe conjugated with serotype-specific capsular polysaccharide subunit (*cpsI*) gene of *S. agalactiae* TP 486 E (serotype III) and species-specific superoxide dismutase (*sodA*) gene of *S. iniae* TSK-2 - via LAMP assay; that offers improved accuracy, and ease of use for the detection of *S. agalactiae* and *S. iniae*, thus aiding to the advancement of molecular diagnostics and its application in aquaculture sector. The LAMP reaction was initially optimised before the AuNPs probe preparation. The hybridisation of the LAMP amplicon and AuNP probe was then optimised for the best visualisation for assay detection. The specificity and

sensitivity of the assay were tested to compare the efficiency of the developed assay with conventional PCR. LAMP amplicons were successfully amplified using the designated LAMP primers. The optimal LAMP reaction condition of the *cpsI* gene in TP 486 E comprises 6 mM $MgSO_4$ with the incubation temperature and time of 65ºC for 60 min, respectively. Meanwhile, the optimal LAMP reaction condition of the *sodA* gene in TSK-2 was 6 mM $MgSO_4$ with incubation temperature and time of 65ºC for 45 min, respectively. The optimal ratio and most distinct colour for visualisation of the assay after salt induction for both strains was at 4:6 of LAMP amplicon: AuNP probe. On the other hand, 0.05 mM of $MgSO_4$ was selected as the best salt concentration for aggregation for both strains. No cross-reactivity was detected in the LAMP assay when tested along the other non-specific aquatic pathogens indicating a high-specificity reaction. The detection limit of bacterial cells for TP 486 E was 4×10^3 CFU/ml, which is 100 times more sensitive than the conventional PCR assay (4×10^5 CFU/ml). The sensitivity of TSK-2 using the LAMP-AuNP assay was 3.3×10^3 CFU/ml, which was 10 times higher than the PCR assay (3.3×10^4 CFU/ml). Both the LAMP-AuNP assay successfully detected the presence of *S. agalactiae* serotype III and *S. iniae* in the infected tilapia juveniles used in the bacterial challenge within approximately 1 hour. The diagnostic sensitivity, specificity and accuracy values for the LAMP-AuNP assay to detect *S. agalactiae* strain TP 486 E compared to the conventional PCR were 100.0, 93.75 and 98.33% respectively. On the other hand, the LAMP-AuNP method for the detection of S. iniae strain TSK-2 yielded 100% diagnostic sensitivity, specificity and accuracy in comparison to PCR assay. There was an excellent overall agreement between the LAMP-AuNP assay and conventional PCR for *S. agalactiae* strain TP 486 E (κ=0.957) and *S. iniae* strain TSK-2 (κ=1.0). The colorimetric detection by LAMP is comparatively similar to the PCR assay but has a shorter turnaround time from extraction to result detection. The developed LAMP-AuNP assay has great potential for use in the detection of pathogenic aquatic *Streptococcus* at the POC and limited resources and settings.

Keywords: Loop-mediated isothermal amplification, aquaculture pathogen, *Streptococcus agalactiae*, *Streptococcus iniae*, gold nanoparticles

SDG: GOAL 14: Life Below Water

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN UJIAN AMPLIFIKASI ISOTERMA PENGANTARA GELUNG BERWARNA UNTUK PENGESANAN SPESIS *Streptococcus* PATOGENIK DALAM AKUAKULTUR

Oleh

NUR MUHAMAD SYAHIR BIN ABDUL HABIB

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Dianggap kuat daya tahan pada mulanya terhadap kebanyakan jangkitan bakteria, wabak di kolam akuatik menunjukkan bahawa tilapia sememangnya boleh terdedah kepada pelbagai penyakit seperti streptokokosis. Jangkitan oleh spesies *Streptococcus* terutamanya *Streptococcus agalactiae* dan *Streptococcus iniae* telah dilaporkan menyebabkan wabak besar yang menjejaskan pengeluar dan petani tilapia. Pengesanan patogen yang pantas dan tepat dengan kegunaan *point-of-care* (POC) adalah wajib untuk mengurangkan kerugian akibat penyakit tersebut. Walaupun tindak balas berantai polimerase (PCR) ialah teknik konvensional yang digunakan secara meluas untuk amplifikasi DNA dalam pengesanan patogen, ia mempunyai beberapa kelemahan seperti kos yanga agak mahal, kerumitan peralatan, memerlukan kitaran suhu yang tepat dan rutin analisis pasca amplifikasi; serta mudah terdedah kepada keputusan positif palsu. Teknik alternatif seperti amplifikasi isoterma pengantara gelung (LAMP) secara amnya menawarkan alternatif yang lebih mudah dan lebih kos efektif, khususnya dari segi keperluan peralatan, kemudahan operasi dan mengurangkan risiko positif palsu. Ciri ini menjadikan LAMP satu pilihan yang baik untuk ujian lapangan dan aplikasi POC di mana amplifikasi asid nukleik yang cepat dan boleh dipercayai adalah penting. Walaupun kelebihan LAMP dari segi kemudahan, kepantasan dan kepekaan, masih terdapat jurang dalam pembangunan ujian LAMP khusus yang dicipta untuk mengesan patogen tertentu dengan tepat dan boleh dipercayai untuk pengurusan dan pengawasan penyakit. Kajian ini berhasrat untuk menangani jurang ini dengan mereka, mengoptimumkan dan mengesahkan alat pengesan kolorimetrik khusus yang menggunakan prob nanopartikel emas (AuNPs) yang berkonjugasi dengan gen subunit polisakarida kapsul (*cpsI*) yang spesifik serotaip bagi *S. agalactiae* TP 486 E (serotaip III) dan gen *superoxide dismutase* (*sodA*) yang spesifik spesies bagi *S. iniae* TSK-2 - melalui ujian amplifikasi isoterma berasaskan gelung perantara (LAMP) yang menawarkan ketepatan

yang lebih baik, dan kemudahan penggunaan untuk pengesanan *S. agalactiae* dan *S. iniae*, sekali gus membantu kemajuan diagnostik molekul dan aplikasinya dalam sektor akuakultur. Tindak balas LAMP pada mulanya dioptimumkan sebelum penyediaan prob AuNP. Proses hibridisasi amplikon LAMP dan prob AuNP kemudiannya dioptimumkan untuk visualisasi terbaik bagi pengesanan ujian. Spesifikasi dan sensitiviti ujian telah diuji untuk membandingkan kecekapan ujian yang dibangunkan dengan PCR konvensional. Amplikon LAMP telah berjaya diamplifikasi menggunakan primer LAMP yang ditetapkan. Tindak balas optimum LAMP untuk gen *cpsI* dalam TP 486 E terdiri daripada 6 mM $MgSO_4$ dengan suhu pengeraman dan masa masing-masing pada 65ºC selama 60 minit. Sementara itu, reaksi optimum LAMP untuk gen *sodA* dalam TSK-2 ialah 6 mM $MgSO_4$ dengan suhu pengeraman dan masa masing-masing pada 65ºC selama 45 minit. Nisbah optimum dan warna yang paling sesuai untuk visualisasi ujian selepas penambahan garam untuk kedua-dua strain adalah pada 4:6 amplikon LAMP: prob AuNP. Sebaliknya, 0.05 mM $MgSO_4$ telah dipilih sebagai kepekatan garam yang terbaik untuk pengagregatan bagi kedua-dua strain. Ujian LAMP tidak mengesan reaksi silang apabila diuji bersama patogen akuatik bukan spesifik lain yang menunjukkan reaksi spesifik yang tinggi. Had pengesanan sel bakteria untuk TP 486 E ialah 4×10^3 CFU/ml iaitu 100 kali lebih sensitif daripada ujian PCR konvensional (4×10^5 CFU/ml). Sensitiviti untuk TSK-2 menggunakan ujian LAMP-AuNP ialah 3.3×10^3 CFU/ml, iaitu 10 kali lebih tinggi daripada ujian PCR (3.3×10^4 CFU/ml). Kedua-dua ujian LAMP-AuNP berjaya mengesan keberadaan *S. agalactiae* serotaip III dan *S. iniae* dalam tilapia juvenil yang dijangkiti di dalam cabaran jangkitan bakteria dalam anggaran tempoh masa 1 jam. Nilai sensitiviti, kekhususan dan ketepatan diagnostik untuk ujian LAMP-AuNP untuk mengesan *S. agalactiae* TP 486 E berbanding dengan PCR konvensional masing-masing ialah 100.0, 93.75 dan 98.33%. Sebaliknya, kaedah LAMP-AuNP untuk pengesanan *S. iniae* TSK-2 menghasilkan 100% sensitiviti diagnostik, kekhususan dan ketepatan berbanding ujian PCR. Terdapat persetujuan keseluruhan yang sangat baik antara ujian LAMP-AuNP dan PCR konvensional untuk *S. agalactiae* TP 486 E (κ=0.957) dan *S. iniae* TSK-2 (κ=1.0). Ujian LAMP-AuNP yang dibangunkan mempunyai potensi besar untuk digunakan dalam pengesanan pathogen *Streptococcus* akuatik pada POC dan sumber dan tetapan yang terhad.

Kata Kunci: Amplifikasi isoterma pengantara gelung, patogen akuakultur, *Streptococcus agalactiae*, *Streptococcus iniae*, nanopartikel emas

SDG: MATLAMAT 14: Kehidupan di Bawah Air

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Syahir Habib, 2022

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Date: 7 November 2024

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% (v/v)	Percent concentration volume / volume
ºC	Degree celsius
µl	Microlitre
µm	Micrometre
x g	Relative centrifugal force
bp	Base pair
CFU	Colony forming unit
Bst	*Bacillus stearothermophilus*
dNTP	Deoxyribonucleotide phosphate
EDTA	Ethylenediaminetetraacetic acid
et al.,	And friends
fg	Femtogram
M	Molar
mg	Milligram
ml	Mililitre
ng/µl	Nanogram per microlitre
nm	Nanometer
OD	Optical density
pg	Picogram
rpm	Revolution per minute
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
UV	Ultraviolet
V	Voltage

CHAPTER 1

INTRODUCTION

Diseases in aquaculture stem from the interaction between the farmed aquatic organism, the causative agent, and nature (external stressors). Among the pathogenic agents, bacteria appear to be the most reported for the frequent emergence and impediments in the global aquaculture industry (Klesius & Pridgeon, 2011; Pridgeon & Klesius, 2012). In general, streptococcosis is a disease prompted by a group of pathogenic *Streptococcus* spp. The most common host infected by the streptococcosis-causing bacteria is fish (Mishra et al., 2018). The highly-infectious disease is considered to be one of the most devastating diseases in the aquaculture industry based on productivity (morbidity and mortality) and economic losses (Amal & Zamri-Saad, 2011; Klesius et al., 2008; Zamri-Saad et al., 2014). Streptococcosis is a complex disease in fish as it interconnects among the type and stage of the host, immune level, availability of pathogens, and environmental settings (Mishra et al., 2018; Vendrell et al., 2006). Tilapias are considered the hardest-hit species/variants by the outbreak of *Streptococcus* spp. (Al-Harbi, 1994; Anshary et al., 2014; Pradeep et al., 2016; Zamri-Saad et al., 2010). *Streptococcus agalactiae* and *Streptococcus iniae* are often reported to be the most frequent representative of streptococcal species that cause streptococcosis in farmed tilapia (Abuseliana et al., 2010; Amal et al., 2013; Anshary et al., 2014; Jantrakajorn et al., 2014a; Klesius et al., 2008; Musa et al., 2009; Zamri-Saad et al., 2010).

Infectious diseases such as streptococcosis cause considerable losses in tilapia farming and aquaculture. Conventional preventions such as disinfection practices and utilisation of antibiotics may backfire with the introduction of hazardous chemical waste to the aquatic environments and the emergence of antibiotic-resistant *Streptococcus* species which may worsen the original state of the disease outbreak (Mishra et al., 2018). Currently, disease monitoring and rapid diagnosis appears to be promising in improving the protective measures against the disease, even before the onset of the streptococcus outbreak (Adams & Thompson, 2011; Luis et al., 2019; Shaalan et al., 2016). Prompt diagnosis of an expected disease and subsequent treatment plays a significant role in preventing the onset of plausible and lasting complications. The practice may also disturb the transmission of an infectious agent such as *Streptococcus*. These acts offer both aquaculture farmers and entrepreneurs a “buffer period” to carry appropriate measures in controlling and reducing the severity of the impending disease (Assefa & Abunna, 2018; Mishra et al., 2018). Diagnostic tests for most infectious diseases usually focus on detecting the pathogenic source, whose presence directly correlates with the diseases. For instance, detecting GBS or *S. iniae* in aquaculture corresponds to streptococcosis.

1.1 Statement of problems and significance of study

Current diagnosis of bacterial fish diseases has shifted from the conventional culture-dependent towards the newer culture-independent approaches, which significantly concentrate on molecular methods (Austin, 2019). The advent of nucleic acid-based detection as the new gold standard in diagnostic tests could be supported upon their advantage to decrease the extended time window in diagnosing infected subjects. The polymerase chain reaction (PCR) has long been considered as the gold standard method in molecular diagnostics. A typical PCR involves temperature cycling which often relies on sophisticated thermocycler and controlled temperature profiles. Nevertheless, the process of PCR amplification can be time-consuming and requires specialised equipment. Furthermore, the post-amplification steps for result analysis such as the gel electrophoresis or real-time PCR detection may introduce a level of complexity to the overall workflow. PCR amplification also often suffers from the risk of false positives results due to contamination during sample handling (Abdelsalam et al., 2023; Ador et al., 2021; Chapela et al., 2018).

Due to the exponential development of the diagnostic method on a molecular level, certain advancements in nucleic acid amplification tests (NAATs) such as isothermal amplification attract a lot of research interest. The appeal of isothermal NAATs lies in their amplification at constant temperatures, minimal and cheap devices, and their short turnaround times in comparison to a PCR, making them suitable for the application in the field of point-of-care (POC) testing (Biswas & Sakai, 2014; Wong et al., 2018). Among the methods of isothermal NAATs, the loop-mediated isothermal amplification (LAMP) method can be placed as the most frequently used method described in the literature (Becherer et al., 2020). It relies on the self-recurring strand displacement DNA synthesis that is performed by *Bst* DNA polymerase which possesses high displacement activity and a set of three specifically designed primers, which comprise the inner (FIP and BIP), outer (F3 and B3) and loop (LF and LB) primers (Nagamine et al., 2002; Notomi et al., 2000). The hybridisation of the four (or six) primers that target six (or eight) different regions in the targeted nucleotide sequence significantly contributed to the high specificity of the method (Becherer et al., 2020; Biswas & Sakai, 2014; Wong et al., 2018). The LAMP assays benefit from their high tolerance towards PCR inhibitors (Inácio et al., 2008; Notomi et al., 2000), impervious to the presence of non-target nucleotide fragments (Inácio et al., 2008; Kaneko et al., 2007; Wang et al., 2008), and well-functioned without the need of extraction steps (Dugan et al., 2012; Kaneko et al., 2007; Poon et al., 2006).

Eventhough LAMP assays hold great promise with regard to their simplicity, rapidity and sensitivity, the successful development and commercialisation of specific LAMP assays customised to various target pathogens remain crucial. While many existing commercial LAMP assays and kits such as the LoopAmp® kits (Eiken Chemical Company, Tokyo, Japan), MmaxSure™ EZ GBS Detection Kit (Mmonitor, Daegu, South Korea) and HiberGene GBS LAMP kit (HiberGene Diagnostics, Dublin, Ireland) focus on the specific detection of *S. agalactiae*, no

LAMP assay have been developed to detect specific serotype of the said pathogen. This considerable gap in current research lies in the absence of a serotype-specific identification method for *S. agalactiae* employing the LAMP assay. On the other hand, there is no commercial LAMP kits or products that readily available in detecting *S. iniae*. These gaps suggest an unexplored prospect to develop a novel and effective approach that can discriminate between different serotypes of *S. agalactiae*, enabling more nuanced and accurate diagnostics in various settings, such as aquaculture. The development of the LAMP assay for the detection of *S. iniae* may also serves as a stepping stones in the advancement of the species detection in aquaculture.

1.2 Hypothesis

This project aims to design, optimise, and validate a novel and specific colorimetric detection tool using gold nanoparticles (AuNPs) probe conjugated with serotype-specific capsular polysaccharide subunit (*cpsI*) gene of *S. agalactiae* TP 486 E (serotype III) and species-specific superoxide dismutase (*sodA*) gene of *S. iniae* TSK-2 - via LAMP assay. This colorimetric LAMP-AuNP assay can be developed and optimised for the sensitivity and specificity to accurately detect the serotype-specific *S. agalactiae* (serotype III) and species-specific *S. iniae* - two pathogenic *Streptococcus* species reported in the local aquaculture settings. The colorimetric detection method, combined with the isothermal amplification process, will provide a rapid, cost-effective, and visually interpretable means of identifying the pathogenic *Streptococcus* species. The employment of AuNP as the visual indicators presents a promising possibility for the enhancement of the assay sensitivity and visual detection, while eliminating the meticulous post amplification steps and adhering to the POC applications. Furthermore, both the accuracy and efficiency of the colorimetric assay can be demonstrated through its ability to detect even low concentrations of the target pathogens, which enable early disease detection and effective disease management strategies in aquaculture settings. To be precise, the following hypotheses (null and alternative) are conjectured:

Null hypothesis (H_0): The developed colorimetric LAMP-AuNP assay does not offer significantly higher diagnostic sensitivity, specificity and accuracy for the detection of serotype-specific *S. agalactiae* (serotype III) and species-specific *S. iniae* in contrast to the conventional PCR - the gold standard in molecular detection method. Hence, it does not provide a more rapid, cost-effective, or visually interpretable means of identifying these pathogenic *Streptococcus* species.

Alternative hypothesis (H_a): The designated colorimetric LAMP-AuNP assay does improve and demonstrate substantially higher diagnostic sensitivity, specificity and accuracy for the detection of serotype-specific *S. agalactiae* (serotype III) and species-specific *S. iniae* compared to the conventional PCR - the gold standard in molecular detection method. Thus, it provides a faster, cost-

effective, and visually interpretable technique to identify these pathogenic *Streptococcus* species.

Based on the statements above, this study was carried out with the following objectives:

1. To evaluate suitable primer sets and optimised conditions for LAMP assay.
2. To optimise the colourimetric detection through the hybridisation of LAMP amplicon and AuNP probes.
3. To assess the specificity and sensitivity of the LAMP-AuNP assay.
4. To evaluate the performance of the LAMP-AuNP assay on infected red tilapia hybrid juveniles in a controlled laboratory setting.

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