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論文題目	Development of onsite and instrument-free recombinase polymerase amplification for smart molecular diagnosis at species level			
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論文の内容の要旨

Research Content:

Nucleic acid identification tests based on conventional polymerase chain reaction (PCR) are often instrumental in choosing the correct treatment for the infection due to the rapidity, sensitivity and specificity of these tests and can be used for the detection of asymptomatic infections[1], early stage diagnosis and disease relapse[2]. Very interestingly, recombinase polymerase amplification (RPA)-based molecular tools have attracted great interest since their initial publication in 2006[3] and are continually emerging as an elegant method of choice for performing amplification without the need for complex instrumentation. Additionally, sampling methods such as liquid biopsy are a burden to both patients and physicians, and the DNA extraction and purification steps involved in sampling in DNA-based methods increase the time to diagnosis. Direct sampling via an FTA card reduces the risk of contamination and facilitates the transport and long-term storage of the sample at room temperature. In this work, a way to discriminate true-positive results from false-positive and/or negative results generated during the RPA reaction is explored and an FTA card is used for direct sampling of RPA reactions to eliminate the concerns involved with sample contamination as well as the sample preparation steps and a method for species level analysis of RPA products obtained for leishmaniasis disease is also developed.

First, we devised an RPA protocol using *Leishmania* species, belonging to the subgenus *Leishmania* and *Viannia* to detect leishmaniasis infection. Next, we demonstrated a near-to-patient diagnostic tool utilizing an integrated approach of RPA, using Whatman FTA card as a direct sampling tool and body heat as the source of incubation temperature targeting a 360-bp gene segment of the 18S rRNA gene, and one-inch gel electrophoretic system. Next, Micro-Temperature Gradient Gel Electrophoresis (uTGGE)-type device is utilized, which has a

power to distinguish, even a single nucleotide difference between two DNA molecules based on their thermal stabilities.

The schematic of the near-to-patient diagnosis is highlighted in Fig.1. First, the sample can be obtained from the patient and is then spotted onto the FTA card and dried. Next, a 2.0-mm-diameter disc is punched from the FTA card, washed and then subjected to liquid RPA reagents supplied with primers targeted to amplify the target gene fragment. This tube is then held in closed fist for 10 minutes to provide the incubation temperature for RPA using body heat. Although the process was simple, the weak true-positive signals amplified in the presence of a low template load (10 parasite copies) could not be distinguished from a negative reaction (no parasite copies) using fluorescent dye. Therefore, the amplified products from RPA are subjected to rapid and portable gel-based detection using one-inch gel electrophoresis. The expected 360-bp band was clearly obtained with 10^4 copies. However, byproducts of smaller fragment sizes were also obtained in reactions containing 10 or no copies of template parasites, which could be clearly distinguished from weak true-positive signals using our handheld electrophoretic device. Next, 10 min- Micro-Temperature Gradient Gel Electrophoresis (uTGGE)-type device is utilized, which has a power to distinguish, even a single nucleotide difference between two DNA molecules based on their thermal stabilities and uses the same type of 1-inch gel electrophoretic system. The melting profiles are obtained and species identification dots are assigned. Pattern similarity scores are then obtained using computer-aided normalization and used to plot a dendrogram. The results can then be submitted to a public database for treatment assessment programmes in epidemic conditions.

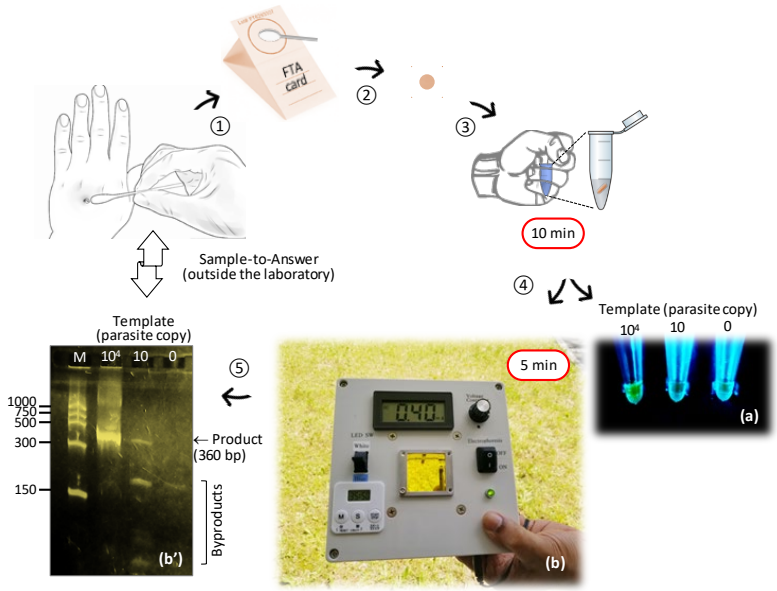

Figure 1: Schematic of near-to-patient nucleic acid-based molecular diagnostic tests.

Research Purpose:

Routine healthcare check-ups are important for early stage diagnosis of the disease. This needs the development of systems to monitor healthcare regularly. Thus, the development of simple, affordable and sensitive point of care testing has become the need of the hour to bridge the gap between diagnostics and treatment of deadly diseases prevailing in today's world. The unavailability of health care resources mainly in the developing world leads to an ever increasing rate of spreading infection from one person to another. However, conventional diagnostic methods such as microscopy, culture-based methods, immunologic tests, non-nucleic acid-based methods and PCR cannot be used in the manufacturing of POCT devices.

In the last decade, a milestone in molecular biology research has been the development of isothermal amplification methods based on some new researches in the molecular biology of DNA/RNA synthesis and their interaction with some accessory proteins. One of the most widely used isothermal amplification methods is recombinase polymerase amplification (RPA) which amplifies the target at 37˚C for the detection of infection in just 10-20 minutes. There have been many publications in the last decade, but these developments have a long way to be available commercially mainly due to the lack of suitable sampling and detection methods as well as generation of non-specific amplification which leads to false-positive results for point-of-care purposes. Detection methods such as flocculation assay detection, electrochemical detection, chemiluminescent detection, silicon microring resonator based photonic detection, surface enhanced Raman scattering detection, etc. are either not suitable for point-of-care and low resource settings or are not able to differentiate between specific and non-specific amplification. Gel electrophoresis is the gold standard for the detection of DNA amplification products, although the tedious steps, time consuming protocol as well as the requirement of transilluminators to visualize the DNA bands renders it unsuitable for POC purposes. Moreover, considering the increasing number of cases of Leishmaniasis (Kala azar) in low resource countries, the current diagnostic methods such as microscopic and culture based detection or Leishmanianin skin test are time consuming and require expensive equipment which are generally available at community centres which are often responsible for 119 villages in India, for instance. Therefore, in this dissertation we engaged to develop a rapid and cost-effective method for the detection of leishmaniasis which can also be used for other diseases just by changing few reagents.

This is the first report of a rapid and portable miniaturized system known as one-inch gel electrophoresis based on the previously developed micro-electrophoretic design to perform onsite polyacrylamide gel electrophoresis in less than 10 minutes. Moreover, FTA card has been used for the first time as a direct sampling tool in RPA reaction to the best of our knowledge. We have used the combination of RPA and one-inch gel electrophoretic system for primary

identification of leishmaniasis infection. However, further we have also engaged to develop a method for species level differentiation of *Leishmania* which is instrumental in diagnosing the different forms of leishmaniasis. The advanced detection utilises the formally developed micro-temperature gradient gel electrophoresis[4] and RPA using 3-4 target gene sequences. In the future, the methods proposed in this research will be evaluated for clinical samples directly for near to patient diagnosis of leishmaniasis. Being a general method and just the need to change the primers for RPA are advantageous as this approach can easily be used for other targets. Our results demonstrate that the combination of robust RPA with FTA card-based direct sampling tools and portable electrophoretic devices can revolutionize nucleic acid-based molecular diagnostics for people in settings with poor healthcare infrastructure.

Research Accomplishment:

Himankshi Rathore, Radhika Biyani, Hirotomo Kato, Yuzuru Takamura and Manish Biyani. Palm-size and one-inch gel electrophoretic device for reliable and field-applicable analysis of recombinase polymerase amplification. *Anal. Methods* (2019). doi: 10.1039/C9AY01476D

Himankshi Rathore, Hirotomo Kato, Yuzuru Takamura and Manish Biyani. Rapid Electrophoretic Typing of Leishmania for Species-level Identification. In preparation.

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Himankshi Rathore, Yuzuru Takamura, Manish Biyani. Development of Smart Wearable 'PCR-on-Paper' Device for Personal Health Monitoring. The 12th Biorelated Chemistry Symposium (CSJ 2016), Osaka, Japan, Sept 7, 2016 *(Poster Presentation)*

Himankshi Rathore, Yuzuru Takamura, Manish Biyani. PCR-on-paper for Wearable DNA Sensing Device using Recombinase Polymerase Amplification and Body Heat. Symposium, University of Toyama, Toyama, Japan, Sept 15, 2016 *(Poster Presentation)*

Himankshi Rathore, Yuzuru Takamura, Manish Biyani. PCR-on-paper for Wearable DNA Sensing Device using Recombinase Polymerase Amplification and Body Heat. Symposium, University of Toyama, Toyama, Japan, Sept 15, 2016 *(Oral Presentation)*

Himankshi Rathore, Yuzuru Takamura, Manish Biyani. PCR-on-Paper for Affordable Personal Health Monitoring using Recombinase Polymerase Amplification and Body Heat. Biyani International Conference 2016 (BICON 2016), Jaipur, India, Oct 20-22, 2016 *(Poster Presentation)*

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Himankshi Rathore, Hirotomo Kato, Yuzuru Takamura, Manish Biyani. Biosensing of Leishmaniasis using FTA card as Direct Sampling Tool for Recombinase Polymerase Amplification. Indian Scientist Association in Japan, Symposium 2018 (ISAJ 2018), Tsukuba, Japan, Dec 7, 2018 *(Poster Presentation)*

Himankshi Rathore, Yuzuru Takamura, Manish Biyani. Versatile Electrochemical Sensor for RPA analysis with Ease. National Institute of Advanced Industrial Science and Technology (AIST) DBT-AIST International Laboratory for Advanced Biomedicine (DAILAB) Workshop 2019, Tsukuba, Japan, Sept 3, 2019 *(Oral Presentation)*

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3 O. Piepenburg, C. H. Williams, D. L. Stemple and N. A. Armes, *PLoS Biol.*, 2006, **4**, 1115–1121.

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論文審査の結果の要旨

本研究は、DNA の等温増幅法である Recombinase Polymerase Amplification (RPA)法と温度勾配ゲル電気泳動法(TGGE 法)を用いて、血液等実サンプルの遺伝子検査を行う方法を確立し、原虫感染症の一種である Lieshmaniasis 症の診断と、その型のある程度の仕分けを、可搬型の装置で可能としたものである。

RPA 法は 40℃の低温で 15 分程度で DNA の等温増幅ができる優れた方法であるが、様々な DNA が混在した実サンプルに応用するとターゲット配列以外の多くの副産物が増幅され、これを防ぐには RPA の前後に機器を用いたそれなりの精製処理が必要であった。本研究は、FTA カードを用いた前処理と小型電気泳動装置を用いた検出が、副産物による擬陽性の抑制に効果的であることを示し、さらにプロテナーゼを用いた後処理等独自の工夫により、RPA の簡便さを損なわずに実サンプルを検査できる一連の方法を確立したものである。

第 1 章では、Lieshmaniasis 症と従来の DNA/RNA 検査法についてまとめ、本研究の課題を明確にした。

第 2 章では、RPA 法により Lieshmaniasis 症を検査するための、効果的な Primer 配列の選定・設計を行い、また primer や増幅時間、濃度の依存性を調べた。これにより RPA は PCR に比べ多くの副産物が得られることが分かり、またそれを最小化する増幅条件を得た。

第 3 章では、電源が得られない Field での検査を想定して、機器を用いずに実サンプルからの感染症診断が可能なプロトコルを開発した。前処理として、FTA カードにサンプルを固定化し、DNA だけを抽出することで、RPA を実施するのに十分な品質の DNA が得られることを示した。また、温調器の代わりにサンプルチューブをアルミホイルで包み、手で温めることで有効な増幅を得た。さらにプロテナーゼを用いた後処理と、別途開発した持ち運び可能な小型の電気泳動撮像装置により、効果的に擬陽性の判別が可能なことを示した。これらにより、大きな機器を用いずに Lieshemaniasis 症を検査できる系を確立した。

第 4 章では、TGGE 法により、Lieshmaniasis 症のサブタイプの仕分けがある程度可能なことを示した。Lieshmaniasis 症は、非常に多くの種類があり、遺伝子配列により仕分けすることは大変である。温度勾配ゲル電気泳動法は、遺伝子配列そのものを調べることなく、遺伝子配列の近さをアナログ的に評価可能である。本研究により、TGGE 法により、Lieshmaniasis 症の内蔵感染型と皮膚感染型の仕分けを含むサブタイプの仕分けを大まかに可能なことを示した。

第 5 章では、本論文の意義を総括した。

以上、本論文は、比較的短時間・低温でDNAの選択的等温増幅が可能なRPA法を実サンプルの感染症診断に応用する際の問題点を明らかにし、その解決策として、電源の得られないFieldにおいても大型の機器を用いずに診断が可能な一連の手法をを発案、確立、実証したもので、学術的に貢献するところが大きい。よって博士（マテリアルサイエンス）の学位論文として十分価値あるものと認めた。