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DETECTION OF MALARIA PARASITES IN LABORATORY INFECTED *ANOPHELES* MOSQUITOES

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Detection of malarial parasites in mosquitoes is important to provide malaria control activities in malaria endemic areas. To establish a best technique laboratory infected mosquitoes are used[1,2]. The mosquito *Anopheles omorii*, *Anopheles stephensi* and the parasite *Plasmodium berghei* were used in the investigations. Among the parasites, *P. berghei* is one of the most commonly studied *Plasmodium* species[3,4].

The experiment was conducted at the laboratory of Department of drug and informatics, University of Okayama, Japan. Both malaria parasite *P. berghei* Anka infected *Anopheles stephensi* and *Anopheles omorii* mosquitoes were received from Jichi Medical School, Tokyo, Japan. The study was targeted for extracting the malaria parasites from the laboratory infected mosquitoes by using the primer pairs targeting the 18s rRNA of *Plasmodium* species.

To conduct the experiment, the mosquitoes used were as blood fed condition (malaria parasite positive) and the other type was non blood fed (malaria parasite negative). Two types of techniques were used for DNA extraction.

Malaria parasite DNA was extracted from *An. omorii* by using grinding buffer method of Collin *et al*. 1987[5] with little modification. The malarial parasite, *P. berghei* Anka infected mosquitoes were preserved in –80°C refrigerator. For grinding, 50 μl of grind buffer was added in the homogenizer and then added 15 μl of 2.5 mg/ml of Proteinase K and the individual mosquito. The homogenizer was kept on ice and grind the mosquito with the pestle about 3 - 4 minutes until no recognizable parts remained. Then the pestle was rinsed by adding 35 μl of grind buffer and transferred the total solution into a new 1.5 ml tube. To denature the nucleases, the solution was incubated at 65°C for 30 minutes and after that added 13 μl of 8 M potassium acetate. Then, the solution was incubated on ice for 30 minutes to precipitate the mosquito parts, other proteins. After the incubation, the solution was spun down and centrifuged at 4°C, with a speed of 14,000 rpm for 15 minutes. Then transferred the supernatant to new 1.5 ml tube and added 200 μl of cold 100% ethanol, mixed well, and left at room temperature for about 5 minutes. Then, centrifuged at 4°C for 20 minutes to pellet the DNA and through the ethanol. After that the pellet was rinsed with 200 μl of 70% ethanol. Finally, the pellet of extracted DNA was dried with a vacuum dryer for about 10 minutes and added 10 μl of distilled water.

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The malarial parasite DNA was similarly extracted from *An. stephensi* as described in case of *An. omorii* except that the DNA extraction kit Fujifilm were used. In that case, the grinded solution of mosquito was transferred into the cartridge of automatic nucleic acid isolation system, Quick Gene-800 and after completing the cycle, got the genomic DNA of malaria parasites.

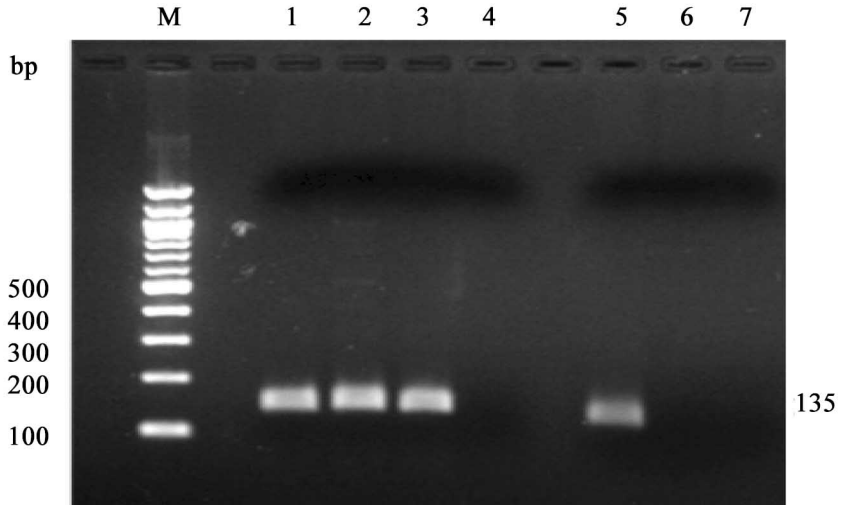

Fig. 1. Electrophoresis result of *Anopheles omorii* infected by *P. berghei*. 1 - 3. Positive control with *P. malariae* DNA with mosquito. 4. Negative control. 5. Malaria parasite positive PCR products from blood fed mosquito. 6, 7. Non-blood fed mosquito.

DNA coding for the small subunit ribosomal RNA (SSUrRNA) is very suitable as a target for the PCR because the SSUrRNA contains the both conserved and variable regions on diverse taxonomic levels. The oligonuleotide primer set, 5′-CAGATACCGT CGTAATCTTA-3′and 5′-CCAAAGACTTTGATTTCTCAT-3′, were described by some workers [6,7] which were specific to the 18S rRNA genes of the human malaria parasites.

For the preparation of DNA for PCR analysis 40 μl of lysis solution was added into new 0.5 ml tube. From the 10 μl of genome DNA of mosquitoes, 1 μl was added at the bottom of the tube. The sample was then incubated at 60°C for 20 minutes, 95°C for 13 minutes to inactivate proteinase K and was cooled at 50°C for about 5 minutes.

For PCR amplification the sample was spun down and mixed with 10 μl of PCR reagent mixture. The mixture was spun down and vortexed and was subjected to 30 cycles. The condition of PCR cycle was as follows: denaturation at 52°C for 60 seconds, annealing at 72°C for 90 second, extensions at 92°C for 90 seconds and holding at 4°C.

The amplified DNA was denatured by heating at 95°C for 10 minutes and then rapid cooling on ice for 10 minutes. Positive control was used as DNA of *P. malariae* from clinical patients and sterilized water was used for negative control. The PCR products

were analyzed by electrophoresis on 1.6% agarose gel (Seakem ME agarose). The gels were run at 100V for 40 minutes. Photographs of the gels were taken using UV transilluminator.

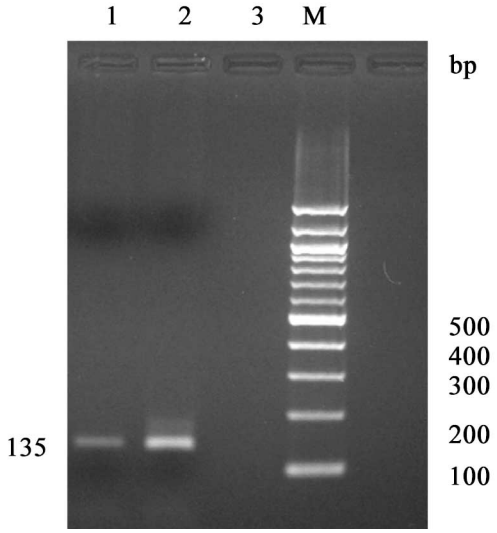

Fig. 2. Electrophoresis result of *Anopheles stephensi* infected by *P. berghei*. 1, 2. Malaria parasite positive PCR products from blood fed mosquito. 3. Negative control.

After the electrophoresis, the result shown that DNA band was appeared from both malaria infected *An. omorii* (Fig. 1), *An. stephensi* (Fig. 2) and not from the non-infected mosquitoes. The DNA band size was same as the DNA band size of positive control of *P. malariae* (Fig. 2). The same results were found where laboratory infected malaria parasites were detected by using nested polymerase chain reaction[2]. It was also investigated that in case of homogenization the DNA extraction from mosquitoes one by one was time consuming that DNA extraction kit and after the electrophoresis it was found that the DNA band was not sharp enough. Therefore, it was revealed that DNA extraction kit were more suitable for DNA extraction than by using grinding buffer and the 18s ribosomal RNA primer pair was specific for detection of malaria parasites in mosquitoes.

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